Screening of an open-source compound library against the livestock parasite *Trypanosoma evansi*

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Abstract

Surra is a neglected tropical veterinary disease that causes a large socioeconomic burden in sub-Saharan Africa, South America, the middle east, Asia, and parts of Southern Europe. Although symptoms can be mild in wild animal species, disease in domesticated species is often acute in nature and can lead to death if left untreated. The causative agent of this disease is *Trypanosoma evansi* (*T. evansi*). *T. evansi* is mainly transmitted mechanically by tabanids (horse-flies) and *Stomoxys* (stable-flies). Current therapeutic treatments for Surra are toxic and fail to treat the disease once the parasite has infiltrated the central nervous system (CNS), particularly, in camels and canines. Previous frontline treatments, suramin and isometamidine chloride, are no longer used in veterinary practice in a number of countries due to widespread resistance. Therefore, it is essential to identify novel or repurposed drugs and compounds that could be used to treat Surra in the future.

This thesis investigates the activity of fertility drug clomiphene citrate against *T. evansi*. The PrestoBlue assay determined that the EC50 of clomiphene citrate against *T. evansi* was 252 nM. Although this value is higher than typical EC50 values for current Surra treatments, clomiphene citrate is already a licensed drug for humans and has the potential to be repurposed. This thesis also investigates the GSK anti-kinetoplastid chemical box against *T. evansi* using a high throughput screening approach. A primary screen at a concentration of 1 µM using the PrestoBlue (AlamarBlue) assay yielded 103 'hit' compounds that were active against *T. evansi* (demonstrated <10% cell viability after incubation for 48 hours). A secondary screen at 100 nM then yielded 13 'hit' compounds. The EC50 values of 9 of these final 'hit' compounds were determined, with 8/9 compounds demonstrating an EC50 <100 nM. The best performing compounds were TCMDC-143326, TCMDC-

143341 and TCMDC-143643 with EC50 values against *T. evansi* of 10.6 nM, 20.4 nM and 12.0 nM respectively. These EC50 values are similar to current treatments for Surra such as diminazene aceturate (8.8 nM).

In summary, clomiphene citrate, as well as several compounds from the GSK anti-kinetoplastid box, demonstrate activity against *T. evansi* with similar *in vitro* effectiveness to current frontline treatments. Further work is required to establish mode of action and cytotoxicity towards specific domestic animal cells.

Contents

I. List of Figures

II. List of Tables

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Chapter 1 – Introduction

1.1 Neglected Tropical Diseases and Trypanosomatidae

A key focus of the World Health Organisation (WHO) is the tackling of neglected tropical diseases (NTDs). NTDs are defined by the WHO as “a diverse group of communicable diseases that prevail in tropical and subtropical conditions in 149 countries.” The diseases predominantly affect impoverished communities and disproportionately affect women and children, causing devastating health, social and economic consequences to approximately 1 billion people. The WHO has recently launched the 2021-2030 roadmap to eradicating or controlling a number of these diseases (World Health Organization, n.d.). These diseases largely affect war-torn or poverty-stricken regions of the globe within populations that lack adequate sanitation, health education and medical facilities. In combination, these diseases are responsible for the deaths of 500,000 people and approximately 57 million disability-adjusted life-years annually – costing billions of dollars (Garchitorena *et al*., 2017).

A significant contributor to the mortality, morbidity and economic burdens of the NTDs is trypanosomiasis. Trypanosomiasis is the collective name given to the group of diseases caused by trypanosomes – unicellular protozoa belonging to the *Trypanosoma* genus of the Trypanosomatidae class of organisms (World Health Organization, n.d.). Trypanosome species can infect humans or animals, both wild and domestic, resulting in severe disease.

1.1.1 African trypanosomiasis

African trypanosomes have severely impacted humans and livestock populations for thousands of years. Egyptian farmers and shepherds suffered due to livestock trypanosomiasis as far back as the old kingdom in 3000-2000 BC (Steverding 2008). African trypanosomes are believed to have separated from other trypanosome

species approximately 300 million years ago. The emergence of the tsetse fly approximately 35 million years ago allowed the parasites to be transmitted via these insects, and also to carry out a significant part of their lifecycle within these vector species (Haag *et al*, 1998;Steverding, 2008). Many African trypanosomes of the *Trypanosoma* genus are dixenous and require the use of both the tsetse fly and mammalian host as a means for a complete lifecycle. This requirement restricts these types of trypanosomiases to regions where the tsetse fly is endemic (as seen in Figure 2) (Kaufer *et al*., 2017).

1.1.2 African trypanosomiasis – *Trypanosoma brucei* (*T. brucei*)

Like many African trypanosomes, *Trypanosoma brucei* (*T.* brucei), use both the tsetse fly and mammals as their hosts to perform their lifecycle as demonstrated in Figure 1.

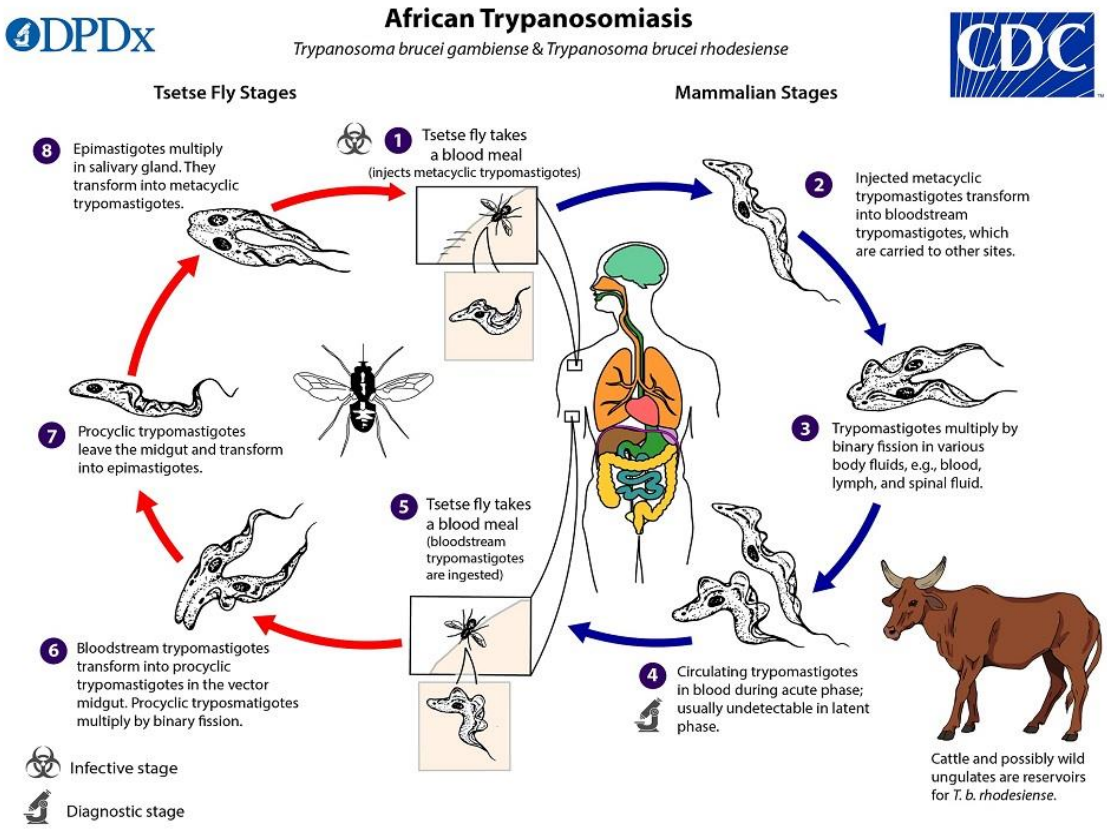


Figure 1* – *African Trypanosomiasis Lifecycle. *A diagram demonstrating the life cycle of sub-species Trypanosoma brucei gambiense and Trypanosoma brucei rhodesiense (CDC, 2019)*

There are three main sub-species of *T. brucei* that cause disease across the tsetse region, each with their own unique differences in endemic areas, mammalian hosts and pathology.

Trypanosoma brucei rhodesiense & Trypanosoma brucei gambiense

Human African trypanosomiasis (HAT) (more commonly known as sleeping sickness) is a disease that primarily affects rural communities in sub-Saharan Africa (Brun *et al*., 2010). The two *T. brucei* sub-species responsible for the disease are *Trypanosoma brucei gambiense (T. b. gambiense) and Trypanosoma brucei rhodesiense (T. b. rhodesiense*.) *T. b. gambiense* is responsible for the disease present in Central and Western Africa and is characterised by a chronic progressive course of the disease. *T. b. rhodesiense* is found in Eastern and Southern Africa and results in acute disease (Migchelsen *et al*., 2011). Infection caused by *T. b. gambiense* are significantly more prevalent than that of *T. b. rhodesiense,* with the former contributing to 97% of current cases (Rijo-Ferreira and Takahashi, 2020). Both diseases can lead to eventual coma and death but the two are preceded by symptoms such as intermittent fever, hepatosplenomegaly and sleep disturbances (Brun *et al*., 2010). In the early stages of disease, the parasites are found within the bloodstream and accumulate in various organs and tissues before breaching the blood-brain barrier and invading the central nervous system (Carvalho *et al*., 2018).

During the 20th century there were several large HAT epidemics affecting countries including Uganda, the Republic of Congo and Cameroon, with the last major epidemic occurring in the late 1990s (Büscher *et al*., 2017). Multiple factors including reduced expenditure and awareness of the disease, social instability, conflict, and immigration/emigration led to the peak in cases during the 1980s and 1990s. The estimated number of HAT cases at the time were reported to be 300,000 cases annually (Franco *et al*., 2014). However, recent control efforts have been successful in reducing the number of recorded cases of the disease. In 2009 the number of cases reported dropped below 10,000 for the first time in 50 years and more recently,

in 2018, there were 977 recorded cases (WHO, 2020). Recent cases are endemic to approximately 20 countries within the tsetse belt region (Büscher *et al*., 2017).

Trypanosoma brucei brucei

Trypanosoma brucei brucei (*T. b. brucei*) is a species of trypanosome capable of causing nagana – a disease specific to livestock animals. The trypanosome is dependent on the tsetse fly for crucial parts of the lifecycle and hence, is restricted to the “tsets belt” as demonstrated in Figure 2. Wild animals are often unaffected by infection or carry only mild symptoms. However, in domestic animals (such as cattle, pigs and sheep) the disease is often fatal if not treated. Many will display symptoms such as fever, listlessness, emaciation, hair loss, discharge from the eyes, oedema, anaemia and paralysis (Steverding, 2008; N’Djetchi *et al*., 2017). Nagana has a significant impact on the local human population who depend on the animals for their dietary products, land cultivation and transport - all of which are limited due to the disease (Connor, 1994).

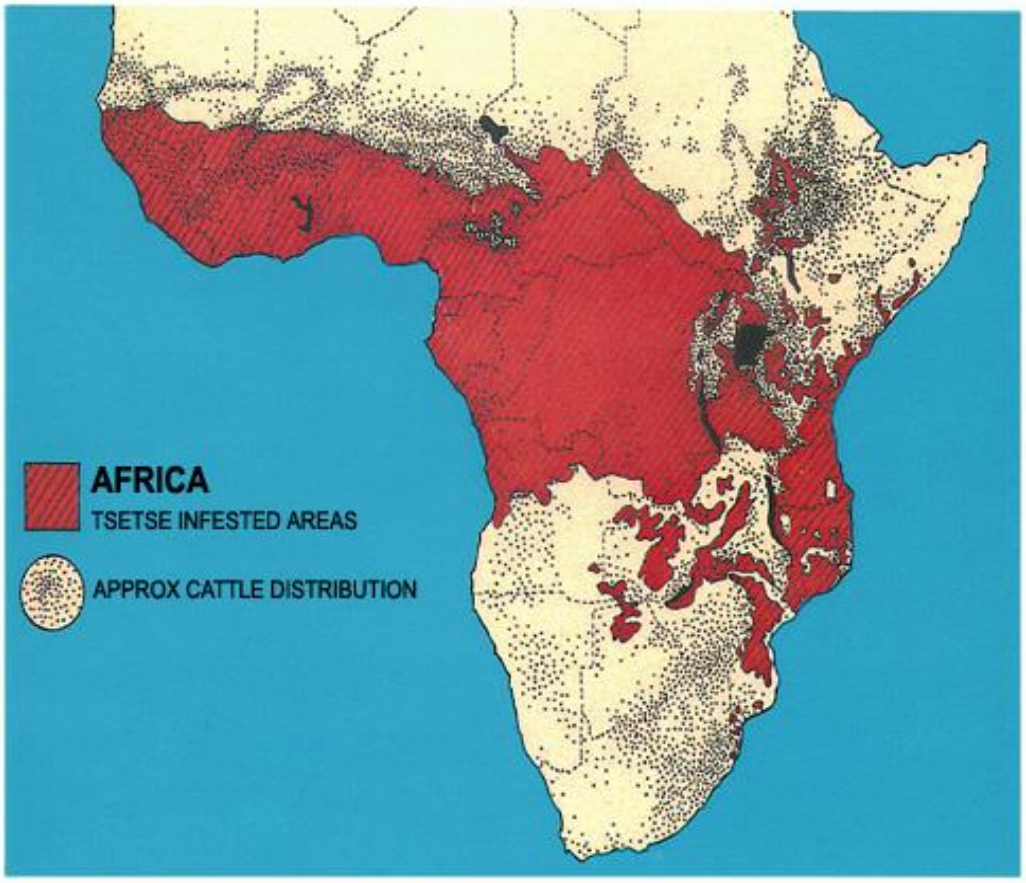


Figure 2 *-* ***A map depicting the areas of tsetse fly infestation as well as the locations of cattle farms****. The tsetse fly is responsible for the deaths of 3 million livestock per year due to trypanosomiasis (PATTEC, 2001)*

1.1.3 Animal trypanosomiasis - *Trypanosoma vivax and Trypanosoma congolense*

Trypanosoma vivax (T. vivax) and *Trypanosoma congolense (T. congolense)* are also capable of causing nagana. *T. vivax* undergoes cyclical development in the tsetse fly and the fly acts as the predominant transmitter of the disease. However, mechanical transmission of *T. vivax* is also possible via the bite of *Stomoxys* and tabanids, establishing the parasite as a non-tsetse transmitted animal trypanosome (Ooi *et al*., 2016). Since mechanical, non-cyclical transmission of the species can also take place, *T. vivax* infections have also been established in South America (Dagnachew and Bezie, 2015). In sub-Saharan Africa, hosts for *T. vivax* include

bovines, equines, caprines, equids and camelids. In Latin America, hosts include bovines and bubalines as well as capybara and wild ungulates (Osório *et al*., 2008). *T. vivax* is the most highly prevalent trypanosome species in African cattle and therefore, plays a large role in the transmission and development of nagana (Ooi *et al*., 2016).

T. congolense is also a key contributor to nagana in sub-Saharan Africa and undergoes cyclical transmission in the tsetse fly, with several differences compared to *T. brucei* (Peacock *et al*., 2012). Like *T. vivax*, *T. congolense* can infect many domestic animal species in East and West Africa including cattle, sheep, goats, horses, camels and dogs (Deschamps *et al*., 2016). The parasites *T. b. brucei, T. vivax and T. congolese* are responsible for the death of approximately 3 million cattle annually at a cost of US$ 1.2 billion in production losses (Chamond *et al*., 2010).

1.1.4 Animal trypanosomiasis - *Trypanosoma evansi*

Trypanosoma evansi is a livestock trypanosome that is believed to have evolved from *Trypanosoma brucei* through the loss of the maxicircles of kinetoplastic mitochondrial DNA (Lai *et al*., 2008). These unicellular, flagellated protozoan parasites cause a type of wasting disease (more commonly known as “Surra” or “Mal de Cadeiras”) that affects mainly livestock, but also domestic animals, predominantly in Africa and Asia. A literature analysis undertaken by Aregawi *et al*., (2019) used 272 references published between 1906-2017 to determine that *T. evansi* is found in at least 48 countries situated in the continents of Africa, Asia, Europe and South America (Figure 3). It is predicted that the geographical range of *T. evansi* may increase with climate change due to an expansion of the optimal breeding temperatures (32-35°C) of its tabanid vector (Reid, 2002).

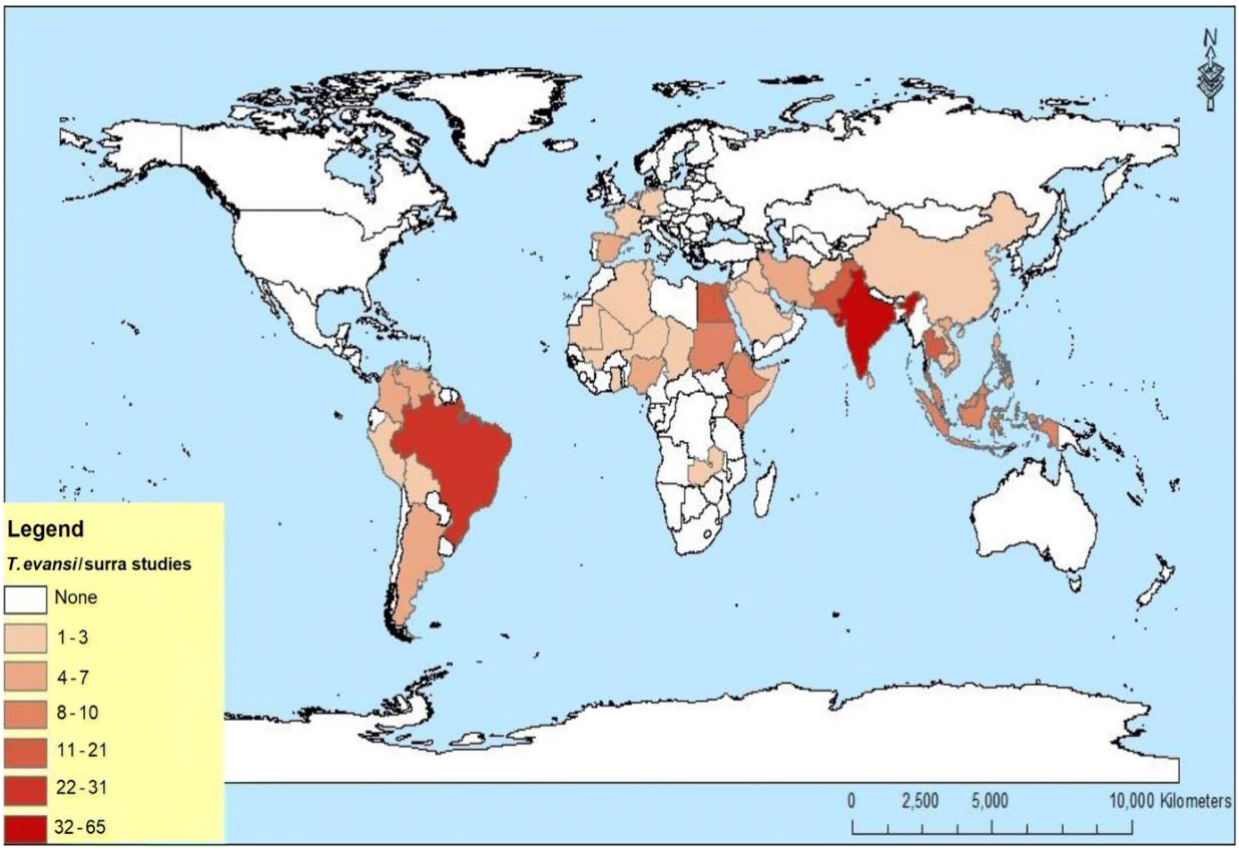


Figure 3 – The estimated global distribution of Trypanosoma evansi using 272 studies published between 1906 and 2017 as reference *(Aregawi et al., 2019).*

T. evansi is indigenous to the African continent and has spread to other continents through the transportation of non-native livestock. In Africa and the Middle East, the main host and reservoir of the parasite are camels but infection can also occur in Equidae, cattle, pigs, domestic sheep, goats, African buffalo (of which it is considered non-pathogenic), domestic cats and dogs (Desquesnes *et al*., 2013). As global temperatures are increasing, areas of the African continent are more reliant on camels as sources of dairy and transportation due to the animal's ability to tolerate harsh weather conditions. Surra represents a significant threat to camel productivity (Diall *et al*., 2022).

In Asia, the water buffalo is very susceptible to *T. evansi* infection, but the parasite also infects horses, cattle, pigs and goats as well as many other mammals (Dargantes *et al*., 2009). In Latin America, it is believed that the parasite was introduced alongside Arabian horses by the conquistadores (Desquesnes, 2004). Here, the disease also has a range of host species including horses, cattle, buffaloes, sheep and goats. Perhaps the most significant wild host of the disease in South America is the Latin American vampire bat (*Desmodus rotundus*). Not only is the bat a host for the disease, but also a reservoir and vector, making it a very important species for the continued maintenance and transmittance of *T. evansi* (Hoare, 1965).

1.1.5 Classification of *T. evansi*

Trypanosoma evansi is classified as follows: Domain: **Eukaryota**, Kingdom: **Protista**, Phylum: **Protozoa**, Subphylum: **Sarcomastigophora**, Order: **Kinetoplastida**, Family: **Trypanosomatidae**, Genus: **Trypanosoma**, Species: ***Trypanosoma evansi*** (Cabi, 2019)

Trypanosomes are diploid organisms and *T. evansi* has a genome size of around 35.2 Megabases (Mb)(Daniels, Gull and Wickstead, 2010; Zheng *et al*., 2019). *T. evansi* is believed to have evolved from multiple *T. brucei* strains, implying that many of the traits displayed by *T. evansi,* such as loss of certain kinetoplast genes and the ability to be mechanically transmitted, have evolved multiple times (Kamidi *et al*., 2017).

T. evansi can also be split into two further categories: type A and type B. Type A is the most abundant of the two and can be found across Africa, Asia and South America. Type B has only been reported in isolates from Kenyan and Ethiopian

camels (Birhanu *et al*., 2016; Ngaira, Olembo, Njagi and Ngeranwa, 2005). The main difference between the two is that type A *T. evansi* isolates are found to have the presence of the variable surface glycoprotein (VSG) RoTat 1.2 gene whereas *T. evansi* type B parasites lack this gene (Birhanu *et al*., 2016). The importance and role of VSGs will be discussed in detail later in section 1.4.

1.2 Transmission of *T. brucei* and *T. evansi*

T. brucei is transmitted by the tsetse fly and the insect is required for essential stages of the lifecycle. This includes the transformation of the bloodstream trypomastigotes into procyclic trypomastigotes and subsequently into epimastigotes. These epimastigotes then transform into metacyclic trypomastigotes after leaving the tsetse's midgut and multiplying in the salivary glands (see Figure 1 and Figure 4) (CDC, 2019). The release of oligopeptides by host and parasite proteins causes *T. brucei* slender bloodstream form to morph into a shorter stumpy form. This form is important for parasite transmissibility into the tsetse fly as well as controlling parasite virulence (Rojas *et al*., 2019). This dependence on the tsetse fly means that *T. brucei* is restricted to the habitat of its vector – the tsetse belt (see Figure 2). In comparison, *T. evansi* has large host and geographical range as the parasite is not reliant on the tsetse fly. Therefore, *T. evansi* has a broader range of transmission methods.

1.2.1 Mechanical Transmission of *T. evansi*

The main mode of transmission of *T. evansi* is through mechanical transmission via a biting insect. This occurs when the insect takes a bloodmeal from an infected mammal and this feeding is interrupted. This interruption is usually initiated through defensive mechanisms of the host such as shaking and swatting of the ears. The insect will then resume its blood meal on another nearby mammal and therefore

transmit the parasite present on the mouthparts. The most common insects involved in this mode of mechanical transmission are tabanids and *Stomoxys,* although many other vectors have been suggested that are more local to certain areas of infection (Desquesnes *et al*., 2013). It is believed that the parasites present on the mouthparts of the insect can infect a new host through the saliva released when the insect initiates a bloodmeal on the uninfected animal (Foil *et al*., 1987). The saliva is secreted into the bloodstream to aid the feeding of the insect due to its anticoagulant properties (Wang *et al*., 2009). This type of transmission is most efficient when the time between the feeding on the first infected host and the subsequent uninfected animals is short. The period in which *T. evansi* is capable of surviving on the mouthparts is extremely short – ranging from 2-7 minutes (Sumba, Mihok and Oyieke, 1998; Oyieke and Reid 2003). The parasite can also survive in the gut of the flies. The period in which *T. evansi* can survive in the gut is significantly longer than that of the mouthparts, with studies demonstrating parasite survival up to 8 hours in a *Stomoxy's* digestive tract (Sumba *et al*., 1998). The insect may regurgitate the contents of its gut whilst feeding, causing the parasites that had survived there to enter the new host (Juyal, n.d.). Due to the short amount of time that parasites remain viable in the mouthparts and gut of insect vectors, mechanical transmission is most effective when animals are in close groups. Not only does this put herds at a high risk of transmission but also allows for interspecies transmission where multiple species are in close contact with each other (Franke, Greiner and Mehlitz, 1994).

1.2.2 Other modes of transmission

Iatrogenic Transmission

T. evansi is capable of being transmitted iatrogenically when a single needle is used for several animals in medical procedures. A study found that this was the case

during a foot and mouth disease campaign in Brazil and Bolivia, potentially increasing the transmission of *T. vivax* and *T. evansi* which are both endemic to the regions (Davila *et al*., 2003).

Transplacental Transmission

Transplacental transmission of *T. evansi* is also a viable mode of transmission. In a study on female Wistar rats infected with *T. evansi*, 80% of the pups tested positive for the parasite demonstrating that *T. evansi* can be transmitted vertically. The same study however describes how *T. evansi* was not transmitted via the venereal route with 100% of pups stemming from an infected male and uninfected female were tested negative for a *T. evansi* infection (Campigotto *et al*., 2017). Transplacental transmission of *T. evansi* has also been demonstrated in sheep as well as the possibility of infection from lambs drinking milk of infected ewes (Campigotto *et al*., 2015)

Oral Transmission

T. evansi is also capable of being transmitted through the consumption of contaminated meat by susceptible raw meat eaters. This has been demonstrated in mice, dogs and even circus tigers (Mandal *et al*., 2017; Raina *et al*., 1985). It is believed that upon entry to the stomach, the few live parasites that have survived the stomachs acidic environment can penetrate the digestive tract or buccal mucous membrane and enter circulation (Mandal *et al*., 2017).

Transmission by Vampire Bats

In Latin America, *T. evansi* can be transmitted by horse flies but also by the *Desmodus rotundus* species of vampire bat. The oral mucous membrane of vampire bats can easily be traversed in both directions by *T. evansi,* giving the potential for

this species of bat to be a good transmitter of the disease (Hoare 1965). Although horse flies are more abundant in number, vampire bats perhaps present an even more effective transmitter of the parasite due to its ability to not only act as a vector but also as a host. The parasite can survive for around 40 days in the bats compared to a few hours in the horseflies (Hoare 1965).

1.3 Life Cycle and Morphology of *T. evansi*

T. evansi has a similar morphology to *T. brucei.* The two are monomorphic trypanosomes that measure 18-34µm in length and 1.5–2.5µm in width depending on the stage of the growth cycle. The parasite cell possesses a small and subterminal kinetoplast, thin posterior extremity, large undulating membrane, central nucleus and a free flagellum (Desquesnes *et al*. 2013). However, *T. evansi* species have lost some, if not all, of their kinetoplast DNA (kDNA) depending on their strain. The term dyskinetoplastic is used for the loss of some kDNA whereas the term akinetoplastic means there is a loss of all kDNA including maxicircles and minicircles (Ventura *et al*., 2000; Schnaufer, 2010). The kinetoplast is discussed in section 1.3.1.

The *T. evansi* lifecycle is less complex than that of the closely related *T. brucei. T. evansi* has been found to have evolved from *T. brucei* through the loss of genes which contribute to their capacity to replicate their kinetoplast DNA. This includes the loss of gRNA genes which are of importance for a trypanosome to transform into the insect promastigote stage (Lai *et al*., 2008). Therefore, *T. evansi* does not have an insect promastigote stage in its lifecycle and the loss of its maxicircle DNA leaves the trypanosome unable to transform out of the blood stream stage (Jensen *et al*., 2008). *T. evansi* has a similar blood stream stage lifecycle to *T. brucei,* including aspects of morphology and metabolism (Moreno and Nava, 2015).

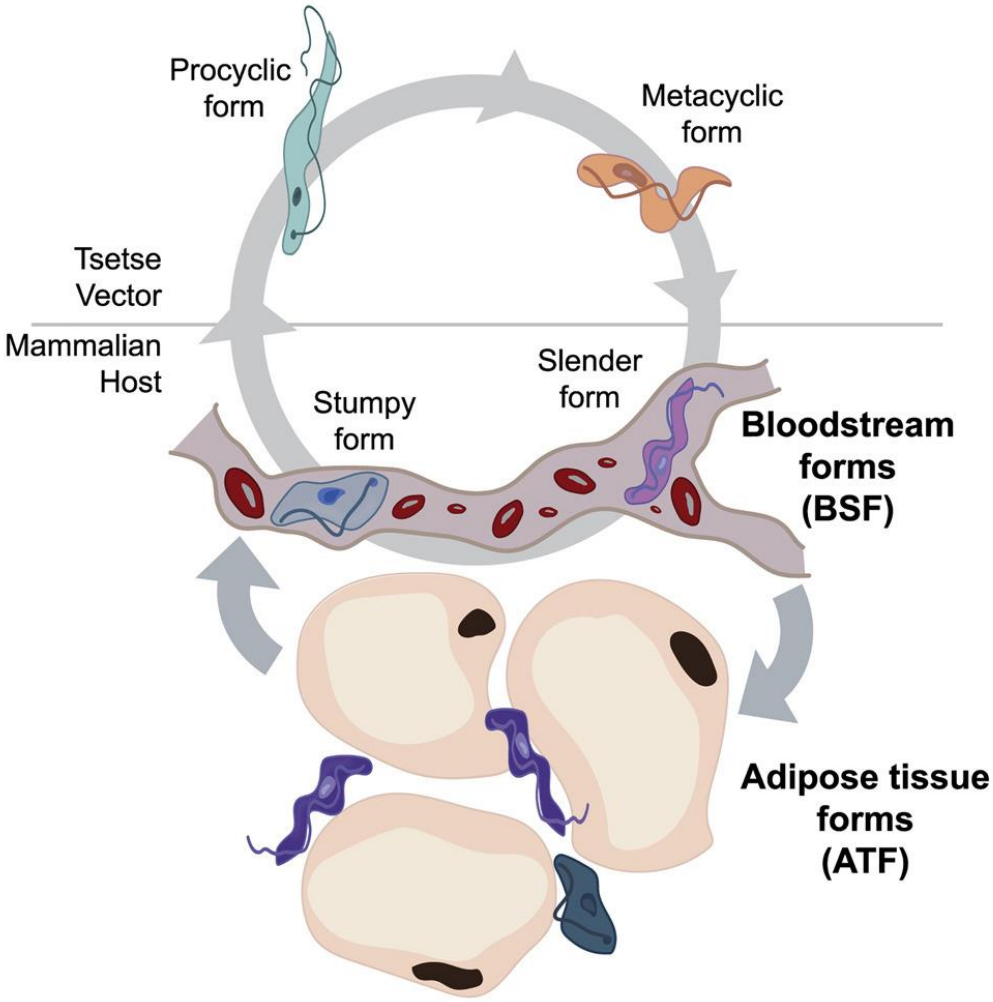


Figure 4 *- **The insect, bloodstream and adipose tissue forms of T. brucei***
(Trindade et al., 2016)

1.3.1 The Kinetoplast

The kinetoplast DNA (kDNA) is a large network of catenated circular DNA molecules, of which there are two types – Minicircles and maxicircles. Maxicircles range from 20 to 40 kilobases in length and are found to encode ribosomal RNAs and some mitochondrial proteins. These topologically locked circles are present in a few dozen copies per network. Minicircles range from 0.5 to 10 kilobases in length and encode small guide RNAs. In comparison to maxicircles, minicircles are much more abundant and diverse, and are present in several thousand copies per network (Lukeš *et al*., 2002; Shapiro 1995). *T. equiperdum*, like *T. evansi*, is believed to have

evolved from *T. brucei* through alteration of the kDNA, however, *T. equiperdum* maxicircles are still present (Li *et al*., 2006).

It is difficult to distinguish between bloodstream form *T. brucei* and *T. evansi* based on morphology alone but a genetic analysis of their kDNA highlights distinguishable differences. The two species have in common a 177 base pair nuclear repeat, amplification of procyclin genes and similar spliced leader gene repeats (Artama, Agey and Donelson, 1992). The main differences between the kDNA of dyskinetoplastic *T. evansi* and *T. brucei* is that *T. evansi* does not possess maxicircles and there are also large differences between the minicircle sequences in the two species. *T. evansi* has minicircles with high homology between strains. A study found that five out of six *T.evansi* strains investigated (four from different regions of Africa and one from South America) differed less than 4% in their minicircle sequence (Borst, Fase-Fowler and Gibson, 1987). In contrast, *T. brucei* has pronounced heterogeneity in minicircle sequence hence, *T. evansi* may have lost this heterogeneity during its evolution from *T. brucei.* Some *T. evansi* strains are akinetoplastic (Ventura *et al*., 2000). As *T. evansi* relies on glycolysis, the presence of the kDNA and the mitochondrial genes within this seem a prerequisite for the parasite's survival. However, the presence of two additional enzymes, phosphoenolpyruvate carboxykinase and PPi-dependent pyruvate phosphate dikinase, aid in the oxidation of glucose in *T. evansi* glycosomes (Rivero *et al*., 2016). It has also been demonstrated that akinetoplastic trypanosomes can survive a loss of kDNA due to a mutation in the gamma sub-unit of the mitochondrial F1 ATP-Synthase (Dean *et al*., 2013). The maintenance of an electrochemical potential across the inner mitochondrial membrane is important for metabolic function. Blood stream form *T. brucei* maintain this potential using the mitochondrial F_1F_0-ATP

synthase complex, with sub-unit a of the complex playing a critical role in the function. The complex functions in reverse to translocate protons from the matrix to the intermembrane space (Zíková *et al*., 2009). *T. evansi* does not possess sub-unit a of the mitochondrial F_1F_0-ATP synthase complex. Hence, a single amino acid change in subunit γ allows *T. evansi* to perform F_0 independent membrane potential generation and maintenance (Dean *et al*., 2013). With the knowledge that *T. evansi* strains can be dyskinetoplastic, or even akinetoplastic, these strains should theoretically be resistant to compounds that target the kinetoplast. It has been demonstrated experimentally that *T. brucei* parasites with no kDNA and an introduced single point mutation in the F1 ATP-Synthase show resistance to diamidine and phenanthridine drug classes (Gould and Schnaufer, 2014).

As kDNA is unique to kinetoplastids and has substantial differences to eukaryotic mtDNA, this makes it a potential target for new therapeutics. However, as shown by *T. evansi,* parasites can retain viability if specific mutations are introduced. This could be a possible pathway of resistance (Jensen and Englund, 2012).

1.4 Immunosuppression and Pathology – *T. evansi*

As *T. evansi* is an extracellular bloodstream and tissue-residing parasite, rapid and effective evasion of the innate and adaptive host immune response is crucial to survival. One of the ways in which *T. evansi* and other related species do this is through the exhibition of variant surface glycoproteins (VSGs). African trypanosomes are believed to hold an arsenal of around 2000 VSG genes, of which, only one is expressed at a time (Pinger, Chowdhury and Papavasiliou, 2017). During infection by a pathogen, a delay occurs between expression of the antigen by the pathogen and development of a functional immune response from the host. During this period, *T.*

evansi, as for *T.* brucei, can switch from expression of one variable membrane surface glycoprotein to that of another. This creates a small sub population that is left untargeted by the immune system that has been directed to attack the parasites expressing the initial displayed antigen (Turner, 2002). This dense proteinaceous coat is believed to be unique to the salivarian group of parasites (Haag *et al*, 1998). Similarly to *T. brucei, T. evansi* VSG consist of a highly variable N-terminal domain of 300-400 amino acids and a far less variable C-terminal domain of <100 amino acids. A single N-terminal domain can form a functional VSG with several different C-terminal domains (Pereira, Jackson and Figueiredo, 2021). VSG is internalised and recycled exclusively by the flagellar pocket. It has been demonstrated in *T. brucei* that the turnover of the VSG cell-surface pool is approximately 12 minutes, and the turnover of the intracellular pool is only one minute (Engstler *et al.,* 2004).

Another way in which *T. evansi* induces immune suppression is through the alteration of NTPDase (Nucleoside Triphosphate Diphosphohydrolase) activity in lymphocytes. It was found that a chronic infection of rats with *T. evansi* led to an increase of NTPDase activity in the host, leading to the downregulation of the inflammatory process through the production of adenosine, a molecule with anti-inflammatory and immunomodulatory functions (Oliveira *et al*., 2012). During the process of adenosine production, NTPDase hydrolyses both adenosine triphosphate (ATP) and adenosine diphosphate (ADP) into adenosine monophophate (AMP) (Zimmermann, 1996). As ATP is known to have an important role in lymphocyte function and the release of cytokines such as interferon-γ (INF-γ) and interleukin-2 (IL-2) from T-cells, this is another way in which an increase in NTPDase further downregulates the inflammatory response (Ralevic and Burnstock, 1998).

The complement system has also been investigated as a means for immunosuppression by *T. evansi.* In a study by Ouma *et al*., (1997) it is found that in a *T. evansi* infection of camels, the initial phase of the infection demonstrates a slight increase in haemolytic complement. However, as the infection progresses, the haemolytic complement activity drops. This is shown to recover after successful treatment. There is therefore a possibility that *T. evansi* can suppress the complement system when parasitaemia is high.

1.4.1 Apolipoprotein L-1

T. evansi can infect many different mammalian species, however, it is extremely rare for the parasite to successfully infect humans. There was a documented case of human infection in a patient with an immunodeficiency in 2006 (Powar *et al*., 2006). More recently, use of combined serological and PCR assays has identified cases of symptomless human *T. evansi* infections in India (Sengupta *et al*., 2022.) As is the case for *T. b. brucei, T. evansi* infection in humans is such a rarity due to trypanolysis of the parasite through high-density lipoprotein particle mediated endocytosis once the trypanosome has entered the blood. The specific lipoprotein responsible for this trypanolytic activity is the primate-specific Apolipoprotein L-1 (ApoL1) (Pays *et al*., 2006). ApoL1 function is achieved through the protein's membrane pore-forming domain that is capable of triggering depolarisation of the trypanosome membrane. This results in an influx of calcium ions that, in turn, causes osmotic swelling of the lysosome and lysis of the trypanosome (Perez-Morga *et al*., 2005). Because of the ability of ApoL1 to protect against trypanosome infections, such as that of *T. evansi,* one possible method of treatment could be through the use of human plasma. In a study involving rats, it was found that treatment with human plasma did increase the longevity, but the treatment did not provide any curative effect (Otto *et al*., 2010). Da

silva *et al*., (2011) also found that treatment with human plasma increased the longevity of mice and, in some instances, the plasma provided curative effects. The inconsistency of the curative effects was attributed to the differing ApoL1 levels in the human plasmas.

1.4.2 Symptoms of Surra

Surra can manifest in a wide range of symptoms and even death in many domestic animal species that become infected. Symptoms in camelids and equines are often acute whereas for African and South American Bovinae, symptoms can be mild, chronic or asymptomatic (Desquesnes *et al*., 2013). In Asia, a Surra infection in domestic cattle can often become acute in nature and lead to death (Reid, 2002). Other domestic animals in which infection with *T. evansi* can lead to acute disease are canines (Rjeibi *et al*., 2015), Asian elephants (Dev Moudgil and Das Singla, 2022) and deer. Similarly, Nagana also causes a range of symptoms and death in domestic animals and symptom severity can differ – even between members of the same genus. For example, The *Bos taurus* cattle native to West Africa demonstrate a higher trypanotolerance than *Bos indicus* and the European *Bos taurus* breeds that were introduced to trypanosomiasis endemic regions much later (Ugochukwu, 2008).

Anaemia

African trypanosomiasis infections can cause anaemia and the process of reduced red blood cell number occurs in distinct phases. In the early acute stage, following peak parasitaemia clearance, there is a dramatic decrease in red blood cell number observed. Red blood cell numbers increase during a recovery phase before again progressively decreasing as the disease enters the chronic phase (Stijlemans *et al*., 2018). Anaemia in African trypanosomiasis could be due to several mechanisms

including haemolysis, erythrophagocytosis and sialidase which is responsible for cleaving erythrocytes surface sialic acids which leads to erythrophagocytosis (Habila *et al*., 2012).

Oedema

Infections with *T. evansi or T. b. brucei* can also cause the development of oedemas. These oedemas can be found in the lungs, heart, face, cornea, legs and abdomen. (Bal *et al*., 2012;Da Silva *et al.,* 2010;Laha and Sasmal, 2007). These can be caused by the production of peroxides and free radicals from an oxidative stress reaction due to the presence of trypanosomes. The aggregation of macrophages and pro-inflammatory cytokines leads to the release of these free radicals and peroxides that can cause damage and inflammation to all surrounding tissues (Saleh, Al-Salahy and Sanousi, 2009).

Splenomegaly and hepatomegaly

It is often found that animals with chronic *T. evansi* infection display pronounced splenomegaly and hepatomegaly. An enlarged spleen may be indicative of increased activity of the mononuclear phagocytic system and their destruction of red blood cells leading to the aggregation of large iron deposits in the spleen (Ghaffar *et al*., 2016). Giant cell aggregations and granulomatous lesions may also add to the severe damage done to the spleen (Biswas, Choudhury and Misra, 2001). As the spleen is responsible for several immunological processes (T-cell activation and B-cell differentiation), hematopoiesis and erythrocyte clearing – damage to the spleen can result in disease progression (Dkhil *et al*., 2022).

In the liver, damage can range from mild to severe. Swollen and rounded hepatocytes with vacuolar spaces in the cytoplasm are commonly found (Ghaffar *et*

al., 2016). Antigen-antibody immune complexes deposited in the liver (as well as all other major organs) potentially play a part in inflammation and the destruction of liver cells. In addition to this, the high metabolic demand of *T. evansi* can also lead to hypoglycaemia, leading to starvation of the liver cells (Cadioli *et al*., 2006). Direct damage to the cells by trypanosome enzymes can also occur as well as further self-destruction of Kupffer cells due to the release of their own enzymes when damaged (Dargantes *et al*., 2005). *T. evansi* infection lowers the levels of glutathione (GSH) and catalase (CAT) enzyme as well as elevating the levels of hepatic malondialdehyde (MDA) and nitric oxide (NO) (Dkhil *et al*., 2020). One of the main responsibilities for GSH and CAT is to provide protection against antioxidants (Pizzorno, 2014; Nandi *et al*., 2019). MDA is a toxic molecule that is a product of lipid peroxidation. Its overproduction is an indicator of high levels of free radicals (Marnett, 1999). Although NO can have a range of beneficial functions, NO is also a major source of reactive nitrogen species (Iwakiri and Kim, 2015). With an increase in reactive oxygen and nitrogen species and reduced scavenging by cells protective molecules and enzymes, damage to cells in the liver are further increased.

1.5 Diagnostics – *T. evansi*

Diagnosis of a *T. evansi* infection can be achieved through several different techniques. This includes parasitological based methods, antibody-based methods and molecular based methods. The technique used is dependent on several factors including cost, available personnel and facilities.

1.5.1 Parasitological – Based Methods

Diagosis of *T. evansi* infections are often carried out using parasitological methods. These methods are based on the visual detection of the parasites through a variety

of techniques, depending on the location of the parasite infection. The parasitological technique commonly used for *T. evansi* infections are smear based. Parasitological based methods are considered to have the lowest sensitivity of all methods used for *T. evansi* infection diagnostics. Aregawi *et al*., (2019) state that studies using parasitological methods report very low prevalence in different species of animal compared to studies that use other methods of detection. The authors theorise that this is because 50-80% of field *T. evansi* infections are chronic and have undetectable levels of parasitaemia in the blood. Both *T. brucei* and *T. evansi* can occupy the adipose tissue around major organs (Pereira *et al*., 2019)

Microscopic examination

Microscopic examination of *T. evansi* is achieved through the staining of a blood smear with Giemsa or an equivalent stain. The method has a low sensitivity compared to molecular based tests (Tehseen *et al*., 2015). Microscopic examination is inexpensive, and the disease can be diagnosed immediately upon observation of trypanosomes if a wet blood film is taken. However, with microscopic examination, it is often not possible to identify which trypanosome is causing the infection unless a thin smear is taken which, in turn, sacrifices the already limited sensitivity of the test. (Uilenberg and Boyt, 1998)

Mouse inoculation

Mouse inoculation is another parasitological method that can be used to diagnose a *T. evansi* infection. The method involves infecting laboratory mice with blood from the suspected infected animals. Repeated blood samples are then taken from the mice over a duration of time and a wet blood film is used for observation of parasitaemia (Monzón, Mancebo and Roux, 1990). Although mouse inoculation is one of the most

sensitive tests to use with the ability to detect a mere 12.5 *T. evansi* parasites per ml at 80% sensitivity, the maintenance of live animals makes it an impractical field diagnostic (Reid, Husein and Copeman, 2001). Mouse inoculation is expensive and ethical justification is difficult to acquire when other effective techniques are available. In addition to this, the method requires a longer length of time than simpler parasitological methods and therefore, use of mouse inoculation is of more use in epidemiological studies than routine diagnostics (Uilenberg and Boyt, 1998).

1.5.2 Antibody-Based Methods

Another method used for the detection of *T. evansi* infections are antibody/based or serological methods. This indirect method of detecting *T. evansi* parasites involves the detection of *T. evansi* specific antibodies or antigens in the blood sample of the subject.

Indirect Fluorescent Antibody Test (IFA)

The IFA test involves washing a smear comprised of an antigen/s from the suspected pathogen with the subject's serum. If the subject is infected with the pathogen, then antibodies will bind to the antigens on the smear. After washing, a second set of antibodies are added to the smear that would not only be able to bind to the subject's antibodies but also produce a fluorescent signal upon doing so. Detection of this fluorescent signal using UV light means that the subject does or did recently have an infection from this particular pathogen (Rupprecht *et al*., 2015). In the case of diagnosing a *T. evansi* infection the *T. evansi* parasite would be used on the smear and diagnosis would be made on the detection of antibodies against the parasite.

The IFA test has a high sensitivity compared to methods such as microscopic examination due to its ability to detect lower numbers of parasitaemia. The sensitivity

for *T. b. rhodesiense* and *T. b. gambiense* infections is 98% (Mudji *et al*., 2020) However, specialist lab equipment and personnel are needed to carry out the diagnosis which is not possible in some regions. It is difficult to obtain some secondary antibodies that are needed for the detection of the subject's antibodies. For example, anti-camel antibodies are not widely available. As the use of whole parasites are used for the test, it is difficult to differentiate between trypanosome species (Uilenberg and Boyt, 1998).

Enzyme Linked Immunosorbent Assay (ELISA)

Like IFA, the ELISA method of diagnosis detects trypanosome specific antibodies present in the blood from an infected animal. The ELISA method differs, using specific antigens rather than using a smear of the whole pathogen. These antigens are coated to the sides of wells in a well plate. In the case of a *T. evansi* diagnosis, a species-specific antigen may be used to get a specific diagnosis (Reid and Copeman, 2003).

However, this along with other serological tests, are unable to distinguish between an active and previous infection because antibodies may stay in circulation for long periods of time (Omer *et al*., 1998). In addition to this, the ELISA can only be carried out in a laboratory setting and the reagents are expensive making it less than ideal for diagnostics in resource poor regions.

Card Agglutination Test (CATT)

The CATT is a test in which trypanosomes/antigens are fixed and stained onto a card and a serum sample is then applied onto the card. The test is positive if the antigen agglutinates. One of the major positives of the test is the ability to carry out

diagnostics both in the laboratory and in the field due to the minimal reagents needed and completion of the test in around five minutes (Abdel-Rady, 2006)

In *T. evansi* infections CATT has a very high specificity and of all the antibody-based diagnostics – is most likely to correctly identify infected animals (DAVISON *et al*., 1999). Depending on parasitaemia, parasitological diagnostic tests may be able to detect an infection earlier than CATT due to the delay in the development of antibodies (Hilali *et al*., 2004).

1.5.3 Molecular Based Methods

More modern techniques used in the diagnosis of Surra are molecular based methods such as PCR. Molecular based methods of diagnostics can rapidly identify microbial sequences using techniques such as Nucleic acid amplification tests and mass spectrometry (Goldenberg, 2013). Molecular based methods have the significant advantage of being able to detect pre-patent and chronic infections which antibody and parasitological methods are not able to achieve (Tehseen *et al*.,2015).

PCR involves the test DNA being denatured and specific primers being used to amplify a section of DNA specific to that species of trypanosome. The procedure is extremely sensitive as only a very small amount of parasite DNA is required, and no radioactive probes are needed. The percentage sensitivity can vary depending on the primers used in diagnostic testing (Fernández *et al*., 2009).

Although PCR testing is argued to be the best in both sensitivity and specificity if completed with suitable primers, its associated cost makes antibody-based studies more attractive for laboratory studies (Tehseen *et al*., 2015).

1.6 Control of Surra

With insufficient data on the number of cases, prevalence and economic impact of Surra, it is difficult to control a disease which is so poorly understood. Nevertheless, there are several ways in which attempts have been made to control the disease and limit its effect on the local livestock and workers.

1.6.1 Economic Impact of Surra

There is little information on the number of global cases of Surra nor an accurate depiction of the economic loss that this may cause. Due to the disease affecting regions of poverty, there is often many cases of misdiagnosis, poor monitoring and non-referral of the animal for veterinary assistance due to cost, poor access or a lack of education on the subject. Although difficult to retrieve accurate numbers, there is no doubt that Surra causes a drastic economic loss to those affected. Government reports in the Philippines from 1989-1997 indicate that an economic loss of around $900,000 occurred in this country due to the disease. This number only considers the worth of the animals whilst alive and not other losses such as decreased resource production and the costs of labour and medication (Manuel, 1998). An outbreak of Surra in Sumba Timur, Indonesia spanning the years of 2010-2016, was found to cause a loss of 1.78 million US $ (Dewi *et al*., 2020). Kumar *et al*., 2017 estimated that Surra causes a total direct and indirect loss of 671.1 million US $ per annum in India. Animal trypanosomiasis is found to lead to a loss of 1-4 billion dollars per year for African farmers alone, who supply many European and North American countries with meat and milk (Chanie *et al*., 2013).

1.6.2 Vector Control

One of the ways used to control Surra is vector control. Simple methods such as clearing up manure and decaying animal food (where stable fly larvae develop) as well as keeping animals inside and away from wooded areas are ways in which livestock can be protected with little to no expense (Foil and Hogsette, 1994).

Both conventional and plant-based insecticides have also been used for the control of *T. evansi* vectors. Stable flies have observed resistance to conventional insecticides including organophosphates and pyrethroids (Cilek and Greene, 1994; Reissert-Oppermann and Clausen, 2019). The use of these insecticides is also problematic due to their toxicity to humans and livestock.

1.6.3 Therapeutic Control

Treatment of *T. evansi* is the most common and effective method of control at the present time. However, treatments for the disease are extremely limited and those trypanocides that are available are associated with frequent and severe side effects. In addition, an increase in widespread resistance to some therapeutics has further reduced the small number of drugs available.

1.6.3.1 Diminazene Aceturate

Diminazene aceturate (DA) (Seen in Figure 5) is one of the most common treatments used for *T. evansi* infections and can effectively treat cattle, buffalo, sheep, pigs and camels (Peregrine and Mamman, 1993). DA is found to have activity against trypanosomes that are otherwise resistant to other therapeutic drugs as well as presenting low incidence of resistance itself (Baldissera *et al*., 2015). DA binds to DNA at AT-rich sites and the binding of the drug to kinetoplast DNA may cause inhibition of replication and the loss of this kinetoplast DNA (Giordani *et al*., 2016).

DA however, is found to be toxic to certain mammals when the dosage is too high and is not effective at single dosages in horses, mules and dogs, with the two former animal species suffering mild to severe toxicity with just a single dose (Tuntasuvan *et al*., 2003). This creates the dilemma of giving the animals a reduced dosage and increasing the risk of not curing the disease, or, giving an increased dosage and increasing the risk of the weakened animal dying to problems caused by the toxicity of the drug (Colpo *et al*., 2005). A study involving mice showed that although treatment given within 3-7 days of infection was curative, treatment given 14 days after infection lead to relapse in the mice (Jennings, Whitelaw and Urquhart, 1977). The authors also suggest that this relapse could be caused by residual parasites 'hiding' in regions of the CNS protected by the blood brain barrier in which DA cannot cross or can only do so in amounts insufficient to kill the trypanosome. Da Silva *et al*., 2009 found that in a previous trial, a single dose of DA (3.5 mg kg^{-1}) did not cure infected felines. However, when treating the felines with 1 dose per day over 5 days, 85.7% of the animals were cured. The study shows that, in some cases, increased dosage of DA can sufficiently kill all *T. evansi* parasites. The felines also showed no effects of toxicity from this increased dosage. A study investigating the use of DA in mice found that combining the treatment with Resveratrol (a polyphenolic compound) reduced the lipid damage on the liver and kidney, perhaps due to the antioxidant properties of Resveratrol (Baldissera *et al*., 2015). Witola *et al*., 2005 describe how acquired resistance to DA is associated with the upregulation of TeDR40 in drug-resistant parasites.

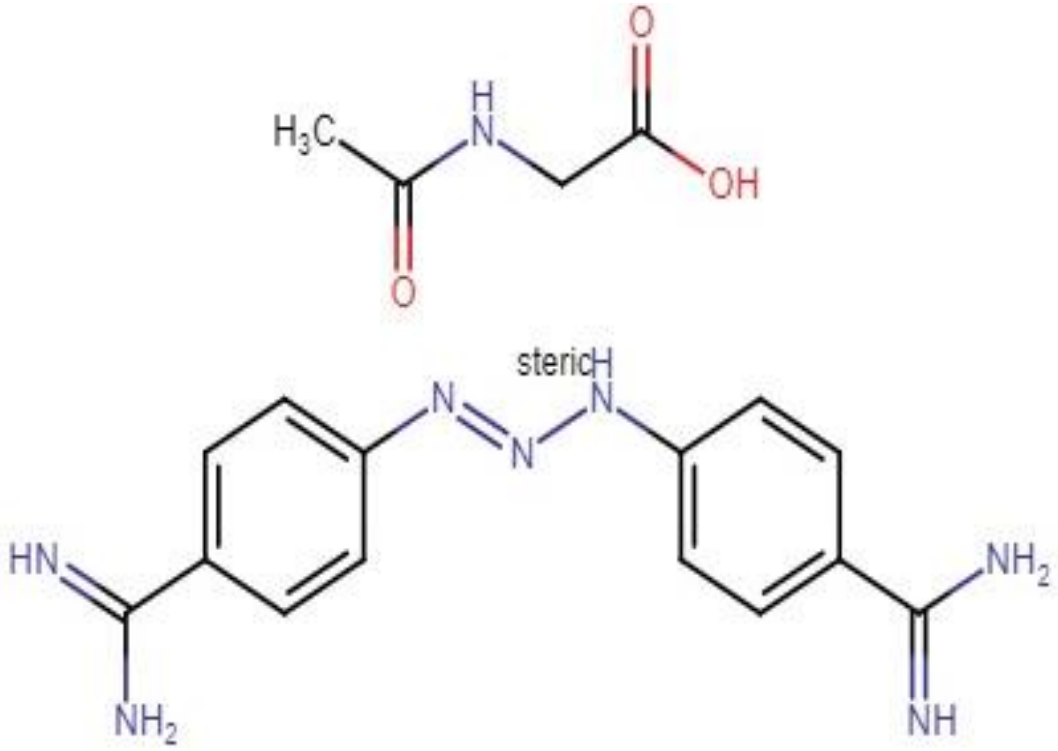


Figure 5 - ***The structure of diminazene aceturate***

1.6.3.2 Melarsamine Hydrochloride

Melarsamine hydrochloride (commercialised as Cymelarsan, see Figure 6) exhibits trypanocidal activity through the inhibition of pyruvate kinase which causes interference in metabolic processes as well as interacting with trypanothione (S. Rathore *et al*., 2016). The compound is the recommended drug for clearing *T. evansi* infections in camels. The drug is administered intramuscularly at a dose of 0.25 mg/kg for camels and 0.5mg/kg in cattle and horses (Desquesnes *et al*., 2011; Camoin *et al*., 2019). Similar to other trypanocides, the drug is incapable of parasite clearance in horses, dogs and camels once the infection has reached the central nervous system (Camoin *et al*., 2019).

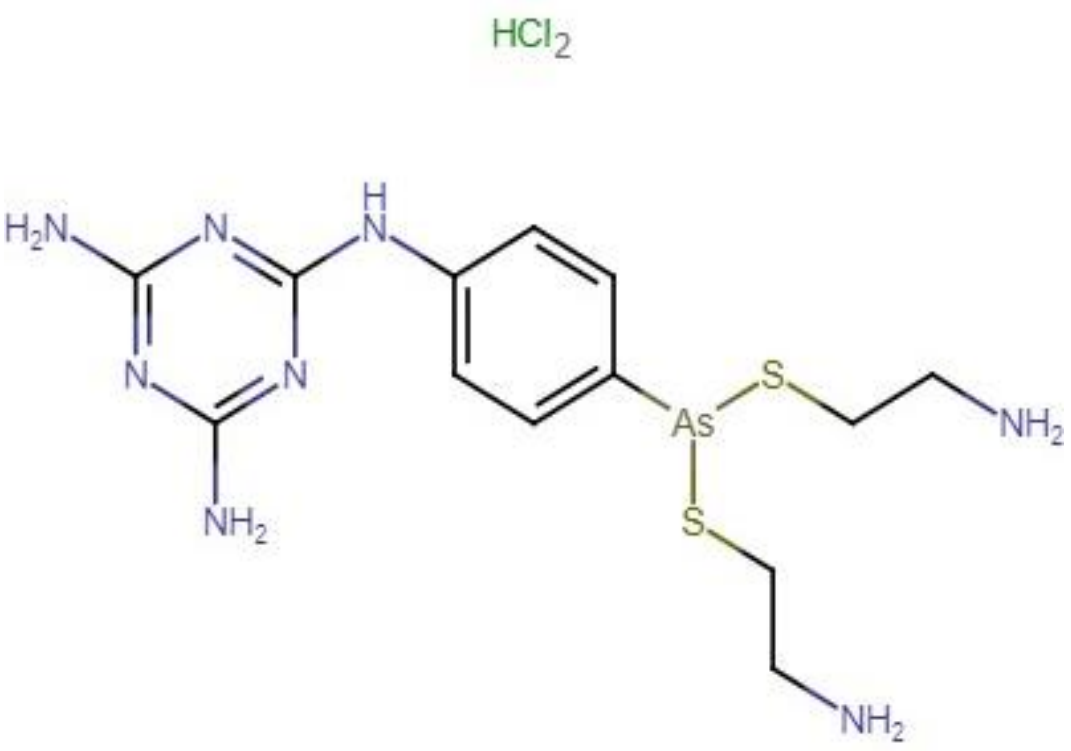


Figure 6 - ***The structure of melarsomine hydrochloride***

1.6.3.3 Isometamidium Chloride

Isometamidium chloride (as seen in Figure 7) is a phenanthridinium drug that is believed to reduce the rate of RNA chain initiation by inhibiting the formation of stable complexes between RNA polymerase and DNA initiation sites (Richardson, 1973). The initial mode of action for ISM was initially perceived to be the inhibition of kinetoplastic topoisomerase type ll. However, it is found that both kinetoplastic and dyskinetoplastic strains of *T. evansi* and *T. equiperdum* are equally susceptible to isometamidium chloride, suggesting that the drug may have another primary mode of action (Kaminsky, Schmid and Lun, 1997).

Both type A and type B *T. evansi* strains in Ethiopia are found to be completely resistant to not only isometamidium chloride but the phenanthridine drug class in

general (Mekonnen *et al*., 2018) as well as some Chinese *T. evansi* strains (Brun and Lun, 1994).

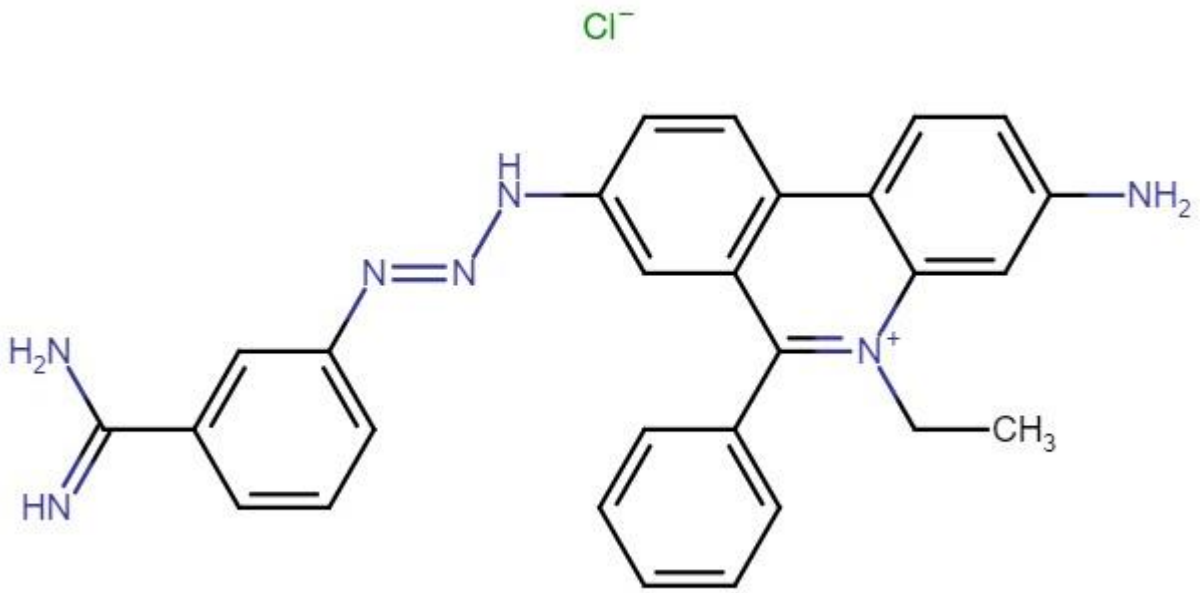


Figure 7 – The structure of isometamidium chloride

1.6.3.4 Quinapyramine Sulphate

Quinapyramine sulphate (seen in Figure 8 is a quinoline pyramidine that is believed to cause trypanocidal activity by blocking nucleic acid synthesis and inhibiting the cytoplasmic ribosome. It is also suggested that the mechanism of action could include mitochondrial accumulation (Giordani *et al*., 2016). Quinapyramine can be administered both intramuscularly and intravenously at a dose of 1ml per 47-67 kg of body weight. The withdrawal times for meat is 21 days and withdrawal time for milk is 4 days (Interchemie, n.d.). Quinapyramine methylsulphate can be used as a curative drug whereas a mix of quinapyrimine methylsulphate and quinapyramine chloride can be used for prophylactic purposes, providing around four to six months of cover (Röttcher *et al*., 1987). The drug can cause severe but transient side effects that

include hypersensitivity reactions, salivation, sweating and tremors (Auty *et al*., 2008).

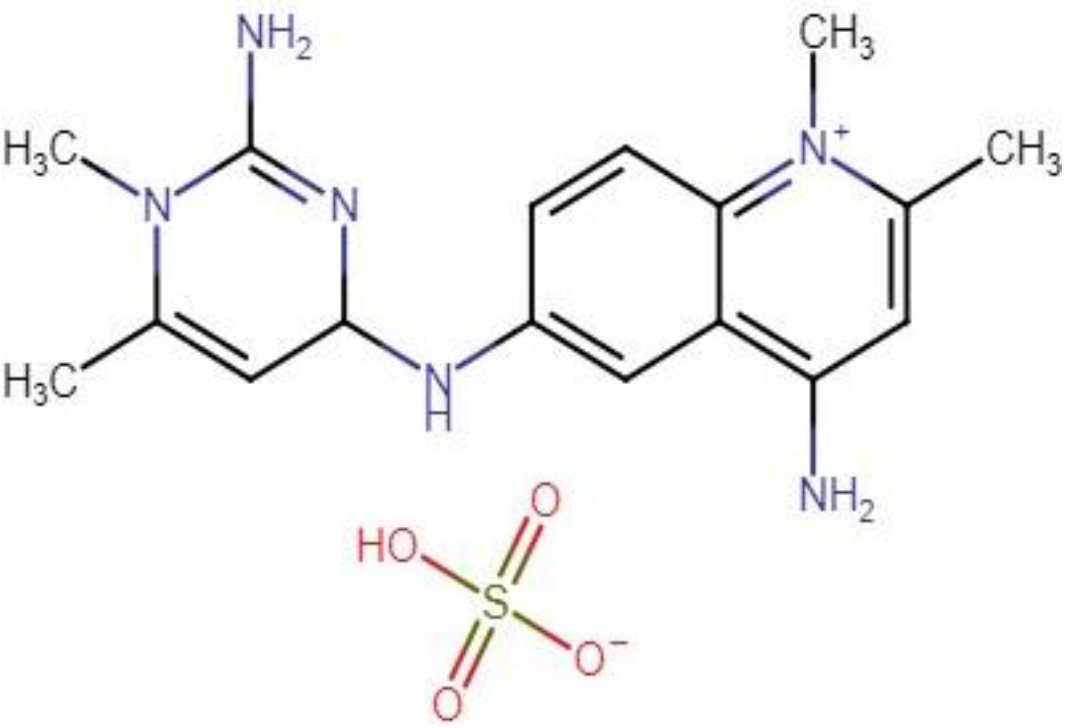


Figure 8 - ***The structure of quinapyramine sulphate***

1.6.3.2 Suramin

Suramin (as seen in Figure 9)is a naphthalene compound that was first used to treat *T. rhodesiense* infections. The drug is found to be effective against both stage 1 *T. b. rhodesiense* and *T. b. gambiense,* however, suramin is only used as a treatment for the former infection (Babokhov *et al*., 2013). The compound is a receptor antagonist that binds to the α- subunit of heterotrimeric G proteins which decreases the speed of interaction between the G protein and coupled receptor (S. Rathore *et al*., 2016). This mechanism disrupts the ability of parasites to carry out glycolysis (Babokhov *et al*., 2013). Suramin is administered intravenously at a dosage of around 12mg/kg per adult camel and excreted slowly which provides prophylactic cover against *T. evansi* for up to 12 weeks in some cases (Röttcher *et al*., 1987). Suramin, like

quinapyrimine, is no longer used in veterinary practice due to widespread resistance to the drug. In a study conducted by El Rayah *et al*., 1999 it was found that even 20 years after its discontinued use in *T. evansi* infections, evidence shows that Sudanese *T. evansi* strains are still resistant to the drug, indicating a stable genotypic characteristic that confers drug resistance in the absence of drug pressure. It is suggested that VSGs could be directly or indirectly involved in suramin uptake and binding in *T. brucei* and that an expression of a specific VSG (VSG^{Sur}) provides resistance to Suramin (Wiedemar *et al*., 2017). Suramin is also ineffective against the second stage of trypanosome infections due to its inability to cross the blood-brain barrier in adequate quantities to kill the parasite (Babokhov *et al*., 2013)

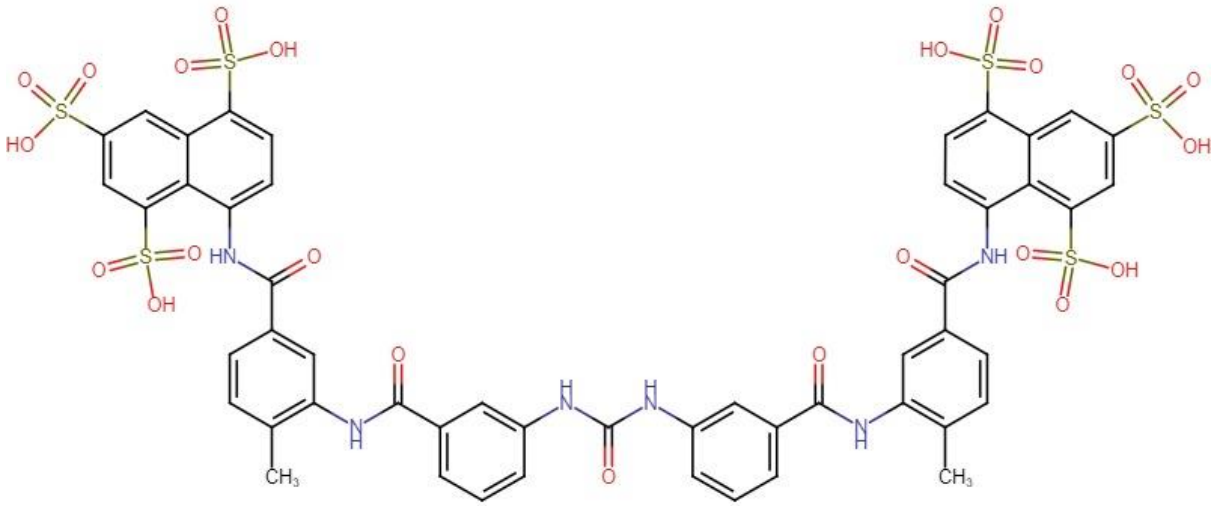


Figure 9 - The structure of suramin

1.6.3.5 Fexinidazole

Fexinidazole (as seen in Figure 10) is the first oral drug for the treatment of *T. brucei gambiense* and is also capable of combatting stage 2 of HAT once the parasites have crossed the blood-brain-barrier. The drug was developed by the Drugs for Neglected Diseases initiative (DNDi) and identified within the nitroimidazole project in 2005 (Torreele *et al*., 2010) (Drugs for Neglected Diseases initiative (DNDi), 2012). The mechanism of action of the compounds involves the inhibition of parasite DNA synthesis (Deeks, 2019). Fexinidazole has already been granted authorisation to be marketed in the Democratic republic of Congo and in July of 2019 was added to the WHO's Essential Medicines List – a list of treatments that the WHO encourages governments to make widely available to populations in endemic countries (Drugs for Neglected Diseases initiative (DNDi), 2019).

Fexinidazole has also demonstrated high efficacy in the curing of *T. evansi* infections in rats (Desquesnes *et al*., 2016). However, an oral drug could prove very problematic for attempted delivery of the drug in larger animals therefore, adequate modifications to mode of delivery would need to be considered. In addition, using a drug for human disease against a veterinary would encourage drug resistance.

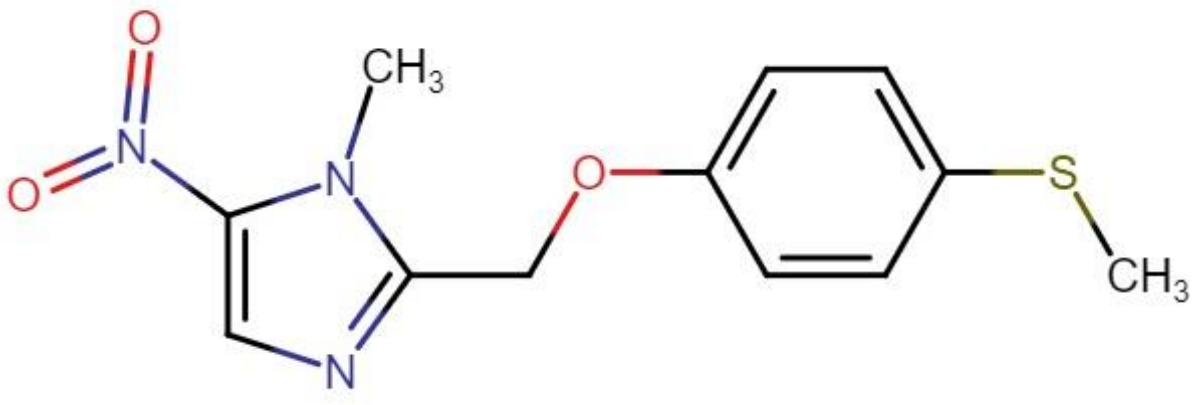


Figure 10 - The structure of fexinidazole

1.6.3.6 Vaccines

There is no vaccine currently available against *T. evansi.* The major problem to the development of a vaccine is the parasite's VSG immune evasion tactic. There have been many attempts to identify vaccine candidates to combat *T. evansi* and *T. brucei* including antigens in the flagellar pocket, transferrin receptors and invariant surface glycoproteins, all of which have demonstrated little success (La Greca and Magez, 2011). A promising potential candidate for a *T. evansi* vaccine is beta tubulin – an integral component of the trypanosome cytoskeleton. Several studies have demonstrated increased survival time and the mounting of a Th-1 response that could be indicative of a viable antigenic target for a vaccine (LI *et al*., 2007;Tewari *et al*., 2015) although further research is needed to portray the true potential of these results. One of the possible issues with this target is that beta tubulin is highly conserved across eukaryote species (Burns and Surridge, 1990). Autheman *et al*., (2021) used a systematic genome-led vaccinology approach to identify a conserved cell-surface protein localised to the flagellum membrane of *T. vivax* that induced long-lasting protection in mice. This discovery potentially identifies a vaccine candidate for other trypanosome species in the future.

1.7 Drug Discovery

The drug discovery process is usually very long and expensive with a multitude of stages and trials that must be adhered to (see Figure 11). Very few drugs successfully traverse the many obstacles along the way. The process from initial discovery through to approval for marketing can take 12 or more years and on average costs around 985 million dollars (DiMasi *et al*., 2010; DiMasi, Grabowski and Hansen, 2016; Wouters, McKee and Luyten 2020).

The first stage of the drug development process for infectious diseases is to identify hit compounds or small molecules which can successfully kill specific pathogens effectively. There are two main ways to identify such compounds – phenotypic screening and target-based approaches. Unlike target-based approaches, phenotypic screening is not reliant on the knowledge of a specific drug target beforehand. The process identifies desirable or potentially therapeutic effects *in vitro and in vivo,* allowing for later identification of its mechanisms (Aulner *et al*., 2019). The phenotypic approach has only recently been bought to the forefront of research again due to advances in technology. The imaging and cell-based detection methodologies were comparatively inefficient, expensive and yielded a lower throughput than that of target-based approaches that used rational mechanism of action and hypothesis driven approaches (Aulner *et al*., 2019). The methods can identify “hit compounds” that show inhibitory activity to the disease. Afterwards, the pre-clinical development stage ensures the compound is suitable and safe for clinical trials. This involves testing the efficacy, toxicity and pharmacokinetics on animal models as well as engineering the compound in a suitable form for administration. After completion of this stage, the drug can then enter clinical development. Here the effects of the drug are first tested in a specific order: human volunteers, selected

patient populations that suffer from the disorder the drug is targeting and finally on vulnerable subpopulations such as the elderly, children or patients with specific illnesses. Often, pregnant women are excluded from studies due to ethical consideration in regard to potential harm to the foetus (Matsui, 2015). Finally, the drug will be monitored in the post marketing surveillance phase for any long-term effects and minor adjustments to dosages (Keiser, Burri and Stich, 2001).

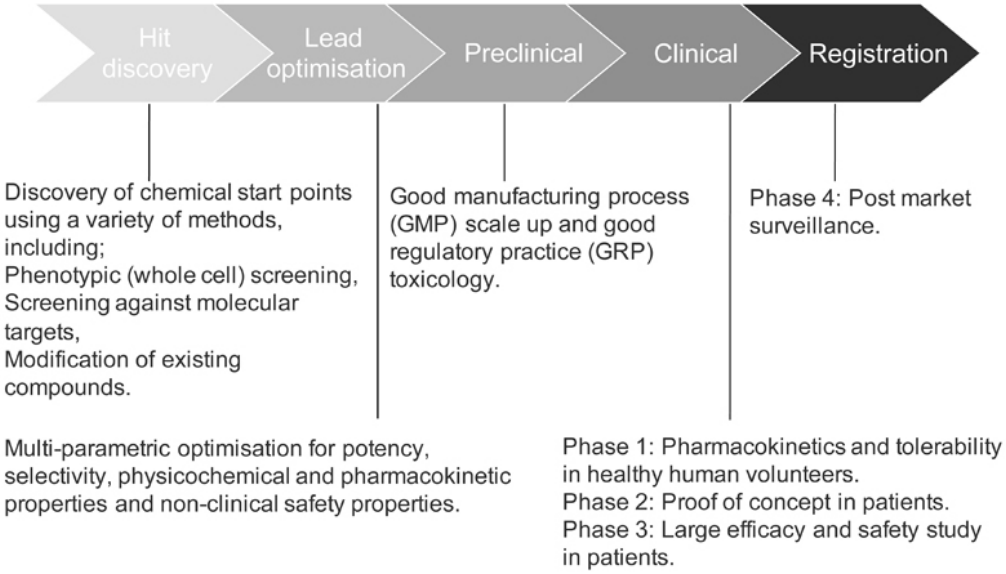


***Figure 11** -**The series of rigorous processes and testing that a compound must pass before being made available on the market** (Field et al., 2017).*

For veterinary treatments, an important evaluation is the drug withdrawal periods for animal-derived products. Firstly, the maximum residue limit (MRL) of the drug in a food product is established by the European Medicines Agency's (EMA) Committee for Veterinary Medicinal Products (European Medicines Agency, 2023). This is established by determining a No Observed (Adverse) Effect Level (NO(A)EL) which is defined as the highest dose that does not cause adverse effects. The figure is then divided by an 'uncertainty' factor to determine the Acceptable Daily Intake (ADI). The ADI is defined as the amount of residue that is considered safe to consume every

day for a lifetime. The withdrawal period is therefore calculated by monitoring the rate of residue depletion to below the MRL in edible tissues and products (milk, eggs, etc.)(Guarav and Sheikh, 2023)

1.7.1 Investigation into Novel and Repurposed Therapeutics for *T. evansi*

With emerging resistance to all therapeutic interventions currently used in the treatment of Surra, as well as the limited efficacy and toxicity of some drugs, the search for novel compounds is important for disease control. No novel therapeutics have been widely implemented in the control of animal trypanosomiasis since the early 1990's. However, several research groups have investigated a wide array of different compound families to find the next potential drug candidate.

There is a strong rationale to repurposing already approved treatments for other conditions and diseases for antimicrobial treatment. Repurposing of 'old' drugs involves the use of 'de-risked' drugs that potentially have lower development costs and shorter development timelines (Pushpakom *et al*., 2018). The WHO recommend the repurposing of therapeutics method of drug discovery for developing new treatments for NTDs. Due to the lack of resources and attention for NTDs new therapeutics must be efficient and have a high rate of clinical success (Klug, Gelb and Pollastri, 2016).

Drug repurposing is already an established method in the repurposing of treatments that show effectiveness in the treatment of HAT. Eflornithine, initially evaluated as a cancer treatment was repurposed as a first line HAT treatment and is one of the few drugs active against the second stage of HAT (Priotto *et al*., 2008). Pentamidine was initially discovered for the treatment of HAT, however, the drug was later repurposed for use in leishmaniasis and treatment of pneumocystis pneumonia. Nassef *et al*.,

(2018) demonstrate the potential for repurposed drugs, demonstrating that cisplatin, an antitumour DNA binding drug, shows activity against *T. evansi* in mice.

It is important to note that drug repurposing does have negative aspects in the context of neglected tropical diseases. Elements such as high cost, toxicity, need for hospitalisation or prolonged treatment and lack of oral bioavailability are large problems in some regions (Field *et al*., 2017)

1.7.2 Target Product Profiles

An important factor to consider whilst identifying new potential treatment is how the compound or drug will fit within the therapeutic target product profile for that disease. Target product profiles (TPPs) provide clear goals and expectations for the drug development process. TPPs are a useful tool for dialogue between regulatory bodies, the many branches within a company and shareholders to highlight what characteristics are expected of a new drug (Breder *et al*., 2017).

The TPP for Therapeutic trypanocide for Animal African trypanosomiasis (AAT), provided by Galvmed (see appendix 3), is relevant for the pursuit of new treatments active against *T. evansi*. As a veterinary disease, there are several unconventional attributes that is needed for the treatment to adhere to. Although many attributes are to be investigated at later stages of drug development, some attributes can be considered at this early stage of compound testing. According to the AAT TTP, the first characteristic relates to the active ingredient of the treatment. Ideally, the agent will be a novel compound that exhibits no cross-resistance to existing treatments. The compound ideally should be able to treat *T. congolense, T. vivax, T. brucei and T. evansi* infections including strains that are resistant to current therapeutics. The treatment should ideally target: Cattle, sheep, goats & other ruminants, camels,

horses, donkeys and pigs. At a minimum, the treatment should target cattle. The target animal should also receive no significant adverse drug reaction and minimal administration site reaction. The treatment should demonstrate safe usage in breeding animals.

The above desired characteristics can be evaluated for some compounds at this early stage of the drug development process. However, this should be considered an initial evaluation to determine which compounds should be pursued further. All profiles mentioned previously should be determined with clear evidence *in vitro/in vivo* and through veterinary trials.

1.8 Summary

There is currently a lack of research and data on *T. evansi* and the impact that Surra has on livestock in all affected countries. This is due to a combination of the chronic nature of Surra, making the disease hard to diagnose, as well as the disease affecting regions of poverty. However, the indirect effects of Surra are severe to the people that depend so greatly on their livestock in affected areas. From inadequate diagnostics to expensive and ineffective treatments, the disease lacks adequate monitoring, control and awareness. Novel treatments need to be investigated, particularly for camel, horse and canine species that suffer from the severe cytotoxic effects of current treatments. For these species, there are no viable treatment when the parasite has invaded the CNS.

The aim of this investigation is to identify novel compounds that demonstrate activity against the livestock trypanosome - *Trypanosoma evansi*. A lack of effective treatments for Surra and growing resistance against current drugs demonstrates the necessity to discover novel treatments. Clomiphene Citrate is a racemic mixture

made up of *cis* and *trans* isomers – zuclomiphene and enclomiphene respectively. A previous study from the Price group has identified the anti-trypanosomal activity of the fertility drug against *T. evansi*. Therefore, this investigation aims to confirm this activity and demonstrate the EC50 of clomiphene citrate using the PrestoBlue assay. The PrestoBlue assay will also be used to demonstrate the activity of compounds from the GSK anti-kinetoplastid chemical box against *T. evansi*. There is no evidence in the literature to suggest that these compounds have been tested against *T. evansi* previously.

Chapter 2 - Materials and Methods

2.1 Materials – Cell Culture and Compound Screening

T. evansi cells (STIB805) were maintained in HMI-9 medium (see Appendix 1) and incubated at 37°C with 5% CO2. The reagents and compounds used in this investigation can be seen in Table 1.

Table 1 - A list of reagents and compounds used in the screening and EC_{50} assays.

Name	Supplier	Product code
PrestoBlue	Thermofisher Scientific	A13261
Anti-kinetoplastid chemical box	GSK	-
Amphotericin B	Thermofisher Scientific	11526481
Dimethyl Sulfoxide (DMSO)	Thermofisher Scientific	J66650.AE
Glycerol	Sigma	G2025-100ML

2.2 Screening of the Anti-kinetoplastid Box

In order to assess the effects of compounds on *T. evansi*, an assay that can monitor and quantify cell viability needs to be utilised. The PrestoBlue (or AlamarBlue) assay has been largely utilised in a range of studies that have investigated cell viability as well as cytotoxicity. PrestoBlue indicates the changes in reducing environment of a living cell. The assay contains the compound resazurin, a blue non-fluorescent dye that is reduced to pink coloured, highly fluorescent resorufin upon accepting electrons (Rampersad, 2012). Only viable cells generate a fluorescent signal as they continue to be metabolically active (Bowling *et al*., 2012).

Screening was performed on the GSK anti-kinetoplastid chemical boxes. The anti-kinetoplastid chemical boxes are composed of three boxes assembled of approximately 200 compounds in each box. Each box contains compounds that are

active against a specific kinetoplastid - *Trypanosoma cruzi, Leishmania donovani* or *Trypanosoma brucei*. There is very little overlap between the chemical boxes (Peña et al., 2015) An initial screen was performed with compounds at a final concentration of 1 µM which were incubated with *T. evansi* cells at a final concentration of $1x10^5$ cells per ml for 48 hours at 37°C with 5% CO_2. A brief overview of the initial screen can be seen in Figure 12.

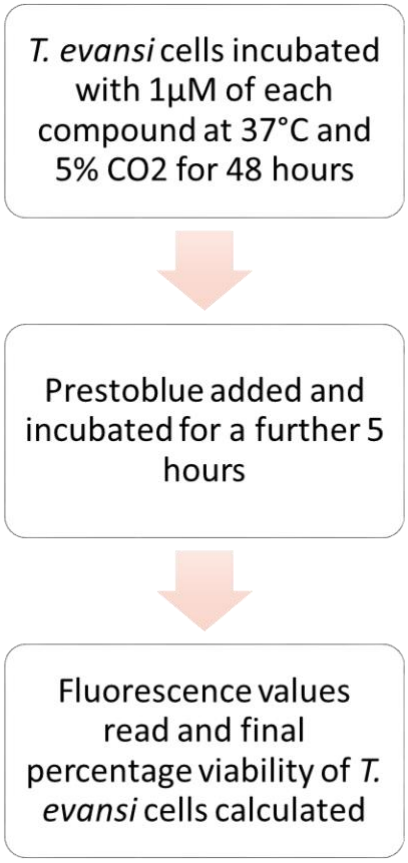


Figure 12 – Demonstration of the the basic process used to perform the initial screen.

Compounds that demonstrated <10% viability in T. evansi cells continued to the next screen.

Compounds were initially diluted in DMSO and therefore, the final concentration of DMSO was 1%. The negative control contained a final concentration of 1% DMSO and $1x10^5$ cells per ml. This control served as the sample which represents uninhibited growth of the *T. evansi* cells and should contain the highest fluorescence reading on the plate. The positive control contained a final concentration of 2.71 µM Amphotericin B and $1x10^5$ cells per ml. This control served as the sample in which complete kill of *T. evansi* cells occurs and therefore should have the lowest fluorescence reading on the plate. The layout of the assay can be seen in Figure 13.

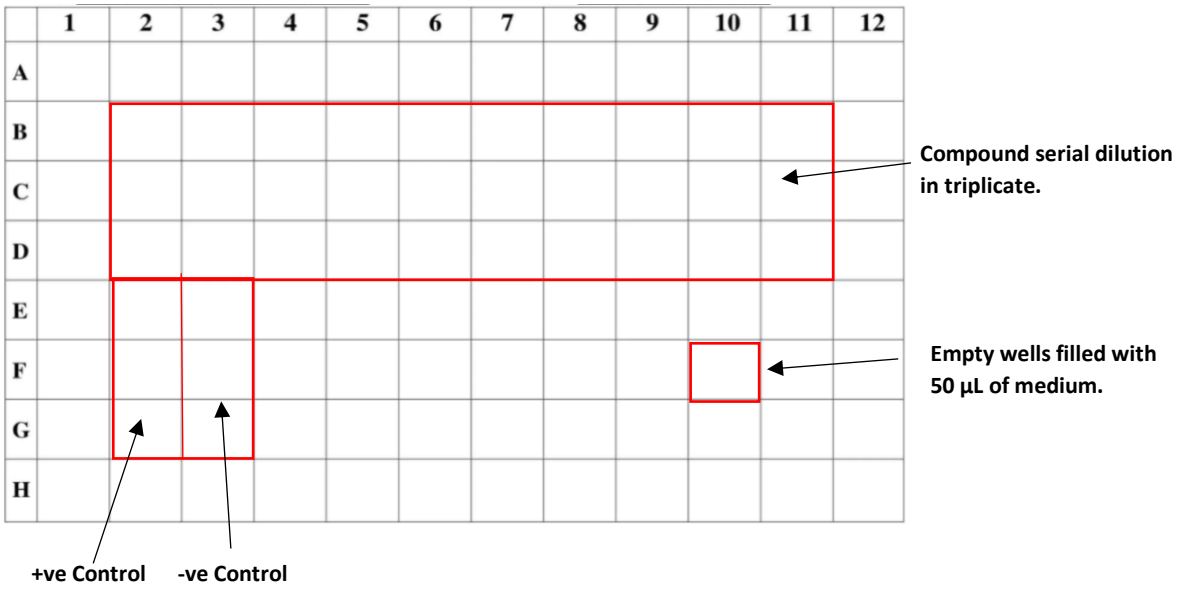


Figure 13 - General layout of the 96 well plate in the EC50 experiment

After the incubation, PrestoBlue was added to each well at a final concentration of 10% and plates were again incubated at 37°C with 5% CO2 for a further 5 hours. Afterwards, the percentage of viable *T. evansi* cells were calculated using the following equation:

$$Percentage\ Viability\ (\%) = 100\ x\ \frac{[\mu(s) - \mu(-)]}{[\mu(+) - \mu(-)]}$$

μ(s) = mean value of sample, μ(+) = mean value of negative control, μ(-) = mean of positive control. Three separate replicate values for each compound concentration were input into the equation and the resulting percentage viabilities were input into GraphPad Prism. The compound concentration was transformed into log10 values and used as the x-axis. The percentage viability values of each concentration were then normalised. The xy analyses was then performed using the non-linear regression (curved fit) function, using the equation: log(agonist) vs normalised response – variable slope. The question investigated was do the best fit values of selected unshared parameters differ between data sets? The data sets were compared using the extra sum of squares F-test and the LogEC50 parameter was selected.

A second screen was performed on compounds that demonstrated a *T. evansi* cell viability of <10% at 1 μM. This screen was performed at a concentration of 0.1 μM using the same methodology as the first screen and as seen in Figure 14.

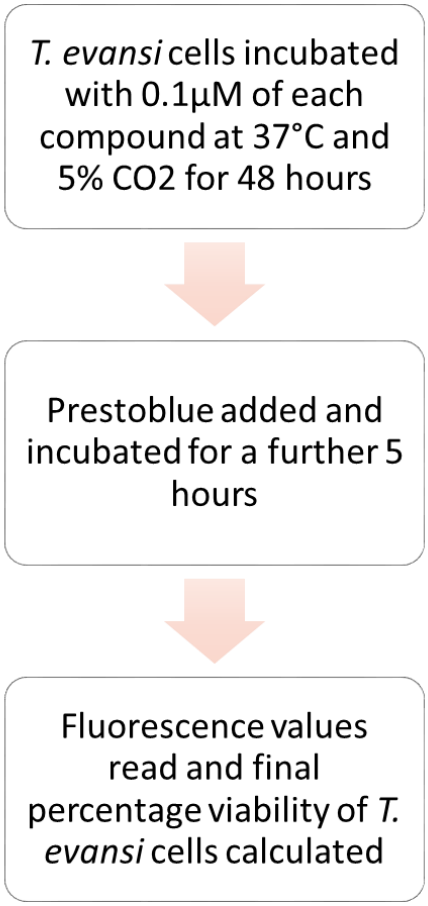


Figure 14 – Demonstration of the basic process used to perform the second screening.

Compounds that demonstrated <10% viability in T. evansi cells continued to the EC50 assay

2.3 Calculating the EC_{50} of Clomiphene Citrate and the anti-kinetoplastid box compounds against *T. evansi*

A stock clomiphene citrate solution of 10 mM was prepared using DMSO. Two-fold serial dilutions of clomiphene citrate were prepared using HMI-9 media with a concentration range of 100 µM – 0.025 µM. The Anti-kinetoplastid box compounds were also serially diluted (two-fold) and the concentration ranges for each compound can be seen in table 1, with a brief overview of the methodology in Figure 15.

To each concentration, 100 µl aliquots of *T. evansi* cells in HMI-9 medium were added - with a final concentration of $1x10^5$ cells per ml. The negative controls for

each compound were made up of a serial dilution of DMSO, matching the same % DMSO as the experimental well, in addition to a final concentration of $1x10^5$ cells per ml. The positive controls consisted of 10 µM of the matching compound and a final concentration of $1x10^5$ cells per ml.

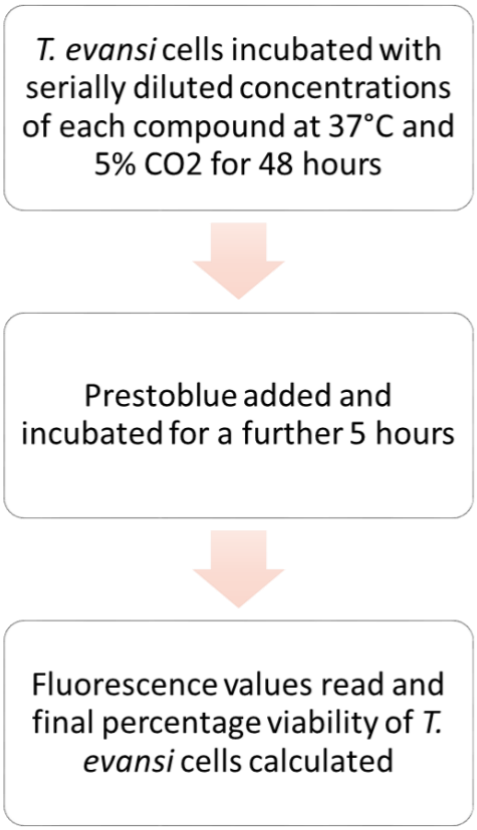


Figure 15 – Demonstration of the basic process used to perform the EC50 assay.

Table 2 - Demonstrates the concentration ranges of each compound in the EC50 assay.

Compound Number	Concentration range for the EC50 experiment
TCMDC-143636	2 µM – 0.0043 µM
TCMDC-143251	
TCMDC-143400	
TCMDC-143289	
TCMDC-143638	
TCMDC-143341	2 µM – 0.002 µM
TCMDC-143363	
TCMDC-143636	2 µM – 0.0011 µM
TCMDC-143643	

2.4 Compound analysis

The simplified molecular-input line-entry system (SMILES) of the GSK anti-kinetoplastid box were obtained from the data sheet provided by GSK (Peña et al., 2015). The SMILES of each compound was input into the Reaxys software to produce a structure. The name of each compound was obtained using PubChem. The SMILES and chemical names of each hit compound can be seen in

Table 3

Table 3 - The chemical name and SMILES of each hit compound

Compound ID	Chemical name	SMILES
TCMDC-143636	N-[4-[(4-pyrazolo[1,5-b]pyridazin-3-ylpyrimidin-2-yl)amino]-2-(trifluoromethyl)phenyl]acetamide	CC(=O)Nc1ccc(Nc2nccc(n2)-c2cnn3ncccc23)cc1C(F)(F)F
TCMDC-143326	2-{4-[1-(1,3-Benzothiazol-2-Yl)-3-Methyl-7-Oxo-4,6,7,8-Tetrahydro-1H-Pyrazolo[3,4-E][1,4]Thiazepin-4-Yl]Phenoxy}Acetamide	Cc1nn(c2NC(=O)CSC(c12)c1ccc(OCC(N)=O)cc1)-c1nc2ccccc2s1
TCMDC-143251	3-(4-chlorophenyl)-2-methyl-5,6,7,8-tetrahydropyrazolo[5,1-b]quinazolin-9(4H)-one	Cc1nn2c([nH]c3CCCCc3c2=O)c1-c1ccc(Cl)cc1
TCMDC-143400	2-[[5-chloro-2-[(2,5-dimethylpyrazol-3-yl)amino]pyrimidin-4-yl]amino]-N-methylbenzenesulfonamide	CNS(=O)(=O)c1ccccc1Nc1nc(Nc2cc(C)nn2C)ncc1Cl
TCMDC-143341	(2S)-1-[4-[(3-Cyanophenyl)methyl]-6-[(5-cyclopropyl-1H-pyrazol-3-yl)amino]pyrimidin-2-yl]azetidine-2-carboxamide	NC(=O)[C@@H]1CCN1c1nc(Cc2cccc(c2)C#N)cc(Nc2cc([nH]n2)C2CC2)n1
TCMDC-143363	N-(5-cyclopropyl-1H-pyrazol-3-yl)-6-methyl-2-(pyrrolidin-1-yl)pyrimidin-4-amine	Cc1cc(Nc2cc([nH]n2)C2CC2)nc(n1)N1CCCC1
TCMDC-143643	2-[3-[(5-bromo-3-methylthiophen-2-yl)methylamino]propylamino]-1H-quinolin-4-one;dihydrochloride	Cl.Cl.Cc1cc(Br)sc1CNCCCNc1cc(=O)c2ccccc2[nH]1
TCMDC-143289	3-(4-bromophenyl)-2-methyl-5,6,7,8-tetrahydropyrazolo[5,1-b]quinazolin-9(4H)-one	Cc1nn2c([nH]c3CCCCc3c2=O)c1-c1ccc(Br)cc1
TCMDC-143638	6-[(4-pyrazolo[1,5-b]pyridazin-3-ylpyrimidin-2-yl)amino]-3H-2-benzofuran-1-one.	O=C1OCc2ccc(Nc3nccc(n3)-c3cnn4ncccc34)cc12

Chapter 3 - Results

The aim of this investigation was to identify novel compounds that demonstrate activity against *T. evansi,* with the goal of assessing some of the most promising compounds to take forward for the development of potential future treatments for Surra. A cell-based assay using PrestoBlue was used to determine the EC_{50} of the most active hit compounds.

3.1 - Optimising the PrestoBlue assay as a compound screening method against *T. evansi*

To conduct cell viability and EC_{50} experiments during this investigation, the resazurin based PrestoBlue assay was used. To optimise the PrestoBlue assay for investigating the test compounds, the assay was first used to determine the EC_{50} value of amphotericin B – a drug with known activity against trypanosomes *in vitro*. 2-fold serial dilutions of amphotericin B concentrations (ranging from 20 µM to 0.039 µM) were added to $1x10^5$ *T. evansi* cells per ml and incubated for 48 hours. Commonly, *in vitro T. evansi* studies use between $1x10^5$-$1x10^6$ cells incubated for 24-48 hours. Amphotericin B will be used as the positive control (0% viability) for the investigative screening. Figure 16demonstrates the EC_{50} curve of Amphotericin B using the PrestoBlue assay. Three experimental replicates were performed with each carried out in triplicate. The standard error of the mean (SEM) is also demonstrated.

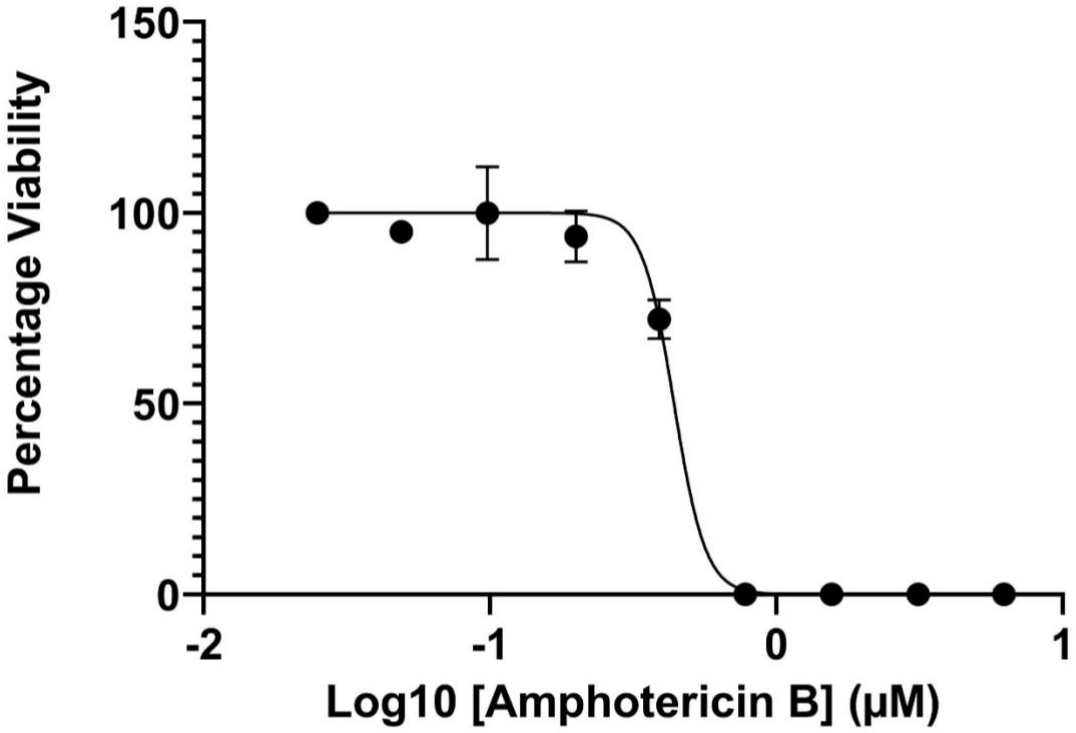


Figure 16 *-* ***EC50 curve of Amphotericin B against T. evansi parasites.*** *Data points for different concentrations of clomiphene citrate were plotted and the* EC_{50} *calculated using GraphPad Prism. The average of the three repeats along with standard error of the mean (SEM) bars are shown.*

As demonstrated in Table 3, the mean EC_{50} value of Amphotericin B was calculated to be 441nM.

***Table 4 - The EC_{50} value for amphotericin B**. Identified using the PrestoBlue assay. The EC_{50} value and confidence intervals were calculated using GraphPad Prism*

Drug	EC50 (nM)	95% confidence interval (nM)
Amphotericin B	441	Unable to calculate

3.2 Results of the anti-kinetoplastid box screening

Although the compounds within the anti-kinetoplastid box have been tested against *T. brucei,* there are no published reports of the compounds being tested on *T. evansi*. The PrestoBlue assay was used to measure the fluorescence of each well resulting in a percentage cell viability figure for each compound tested after a 48-hour incubation period with the parasite. A confirmed hit was defined as a compound that induced less than 10% cell viability, compared to controls, in the *T. evansi* cells after a 48-hour incubation period. All screened compounds and their percentage viabilities are displayed in Figures 16 to 19 and are listed in table format in Appendix 2.

The compounds used had initially been diluted to a 100µM concentration in DMSO. Therefore, the negative control contained a matched DMSO concentration (1%).

3.2.1 GSK anti-kinetoplastid box – Human African Trypanosomiasis (HAT) Compounds

As *T. brucei* is closely related to *T. evansi* it was hypothesised that many of the GSK HAT compounds would show activity against the parasite.

The cell viability data obtained from the compounds in the HAT chemical boxes demonstrate that 80/186 compounds (43%) showed activity against *T. evansi* resulting in less than 10% viability at a concentration of 1 µM as shown by Figure 17.

Due to *T. evansi* and *T. brucei* being very closely related at the genomic level, it is likely that many compounds would be equally effective against blood stream form parasites of both species. In the GSK analysis of the 186 HAT compounds, all compounds had an EC_{50} equal to or below 1µM when investigated for anti *T. brucei* activity. Of the HAT compounds, 38/186 compounds (20%) showed very little to no activity against *T. evansi* (had a calculated cell viability of over 90%) at 1µM.

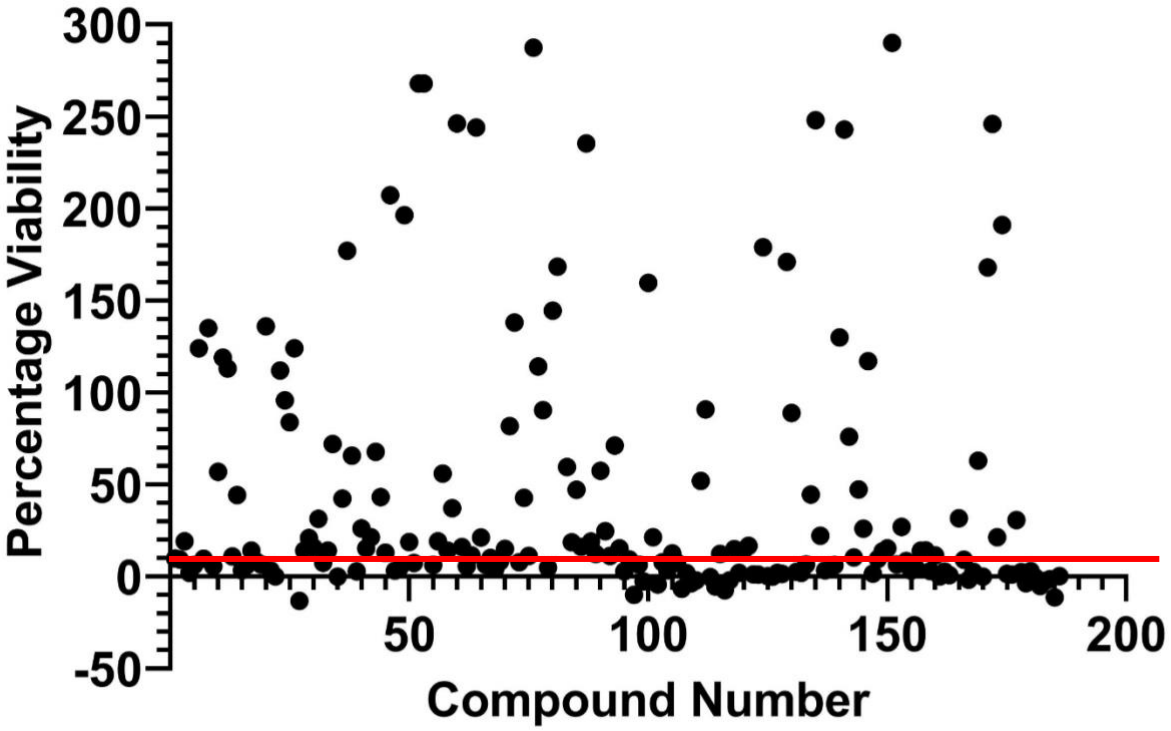


***Figure 17** - **Screening of the GSK HAT compounds against T. evansi at a concentration of 1uM.** Compounds were determined as active if they demonstrated less than 10% percentage viability after 24h incubation (indicated by the red line).*

3.2.2 GSK anti-kinetoplastid box – Chagas Compounds

The GSK Chagas compounds, shown to be effective against *Trypanosoma cruzi*, were also tested for activity against *T. evansi* at a concentration of 1µM. As seen in Figure 18, 12 of the 219 chagas compounds (5%) showed activity against *T. evansi* (a cell viability of less than 10% after a 48-hour exposure).

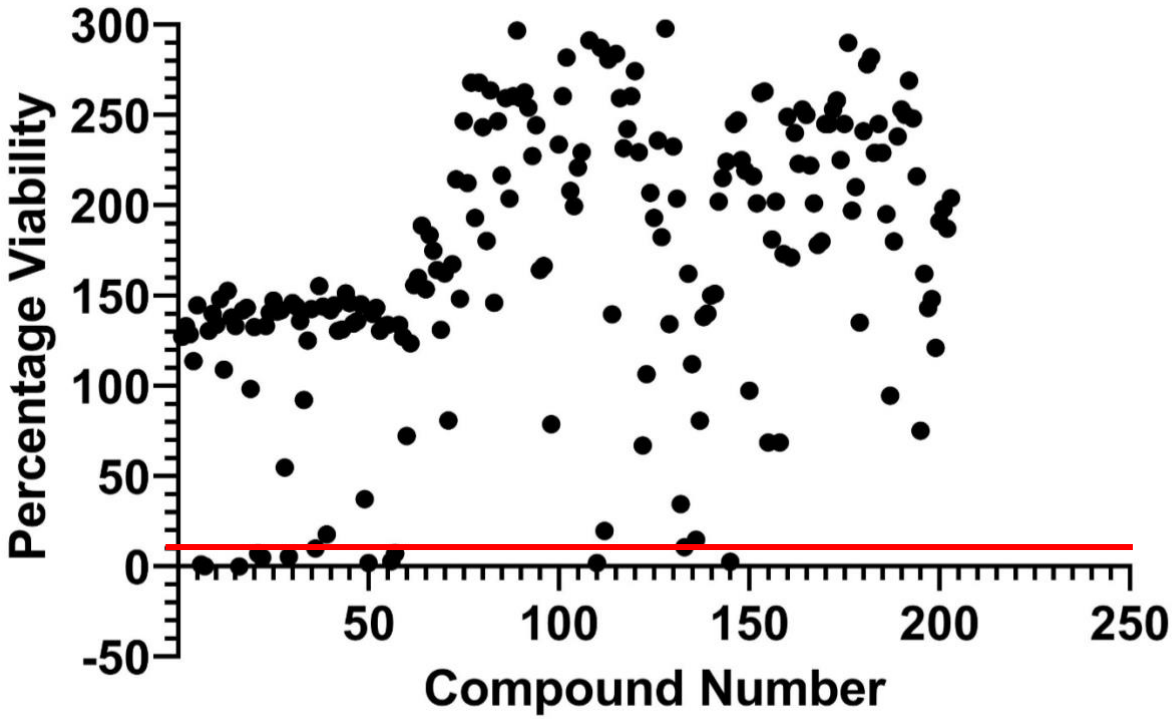


Figure 18 *-* ***Screening of the GSK chagas compounds against T. evansi at a concentration of 1uM****. Compounds were determined as active if they demonstrated less than 10% percentage viability after 24h incubation (indicated by the red line).*

3.2.3 GSK anti-kinetoplastid box – Leishmanasis Compounds

Finally, the GSK leishmaniasis compounds (active against *Leishmania major)* were tested for their activity against *T. evansi* at a concentration of 1 µM. As seen in Figure 19, 11 of the 183 leishmaniasis compounds (6%) showed activity against *T. evansi* (a cell viability of less than 10% after a 48-hour exposure).

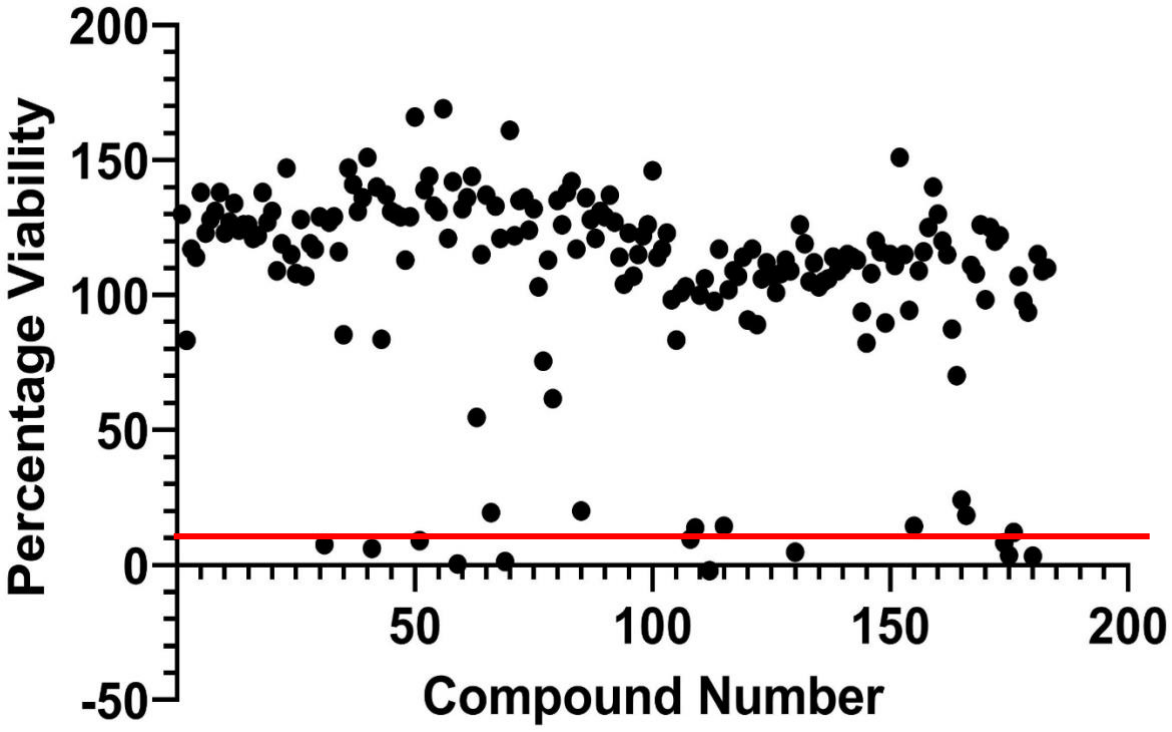


Figure 19 *- **Screening of the GSK leishmaniasis compounds against T. evansi at a concentration of 1uM**. Compounds were determined as active if they demonstrated less than 10% percentage viability after 24h incubation (indicated by the red line).*

3.3.1 Secondary Screen of Compounds

The 103 'hit' compounds from the primary screen demonstrating a cell viability of <10% at a concentration of 1 µM were then screened a second time following the same procedure using a compound concentration of 0.1 µM. This was to narrow down the remaining large compound set from 103 to a few select compounds for further investigation.

As demonstrated by Figure 20, 11 compounds from the HAT chemical box demonstrated a cell percentage viability of less than 10% at 0.1 µM against *T. evansi*. These compounds include - TCMDC-143636, TCMDC-143326, TCMDC-143251, TCMDC-143400, TCMDC-143341, TCMDC-143363, TCMDC-143643, TCMDC-143289, TCMDC-143638, TCMDC-143378 and TCMDC-14352.

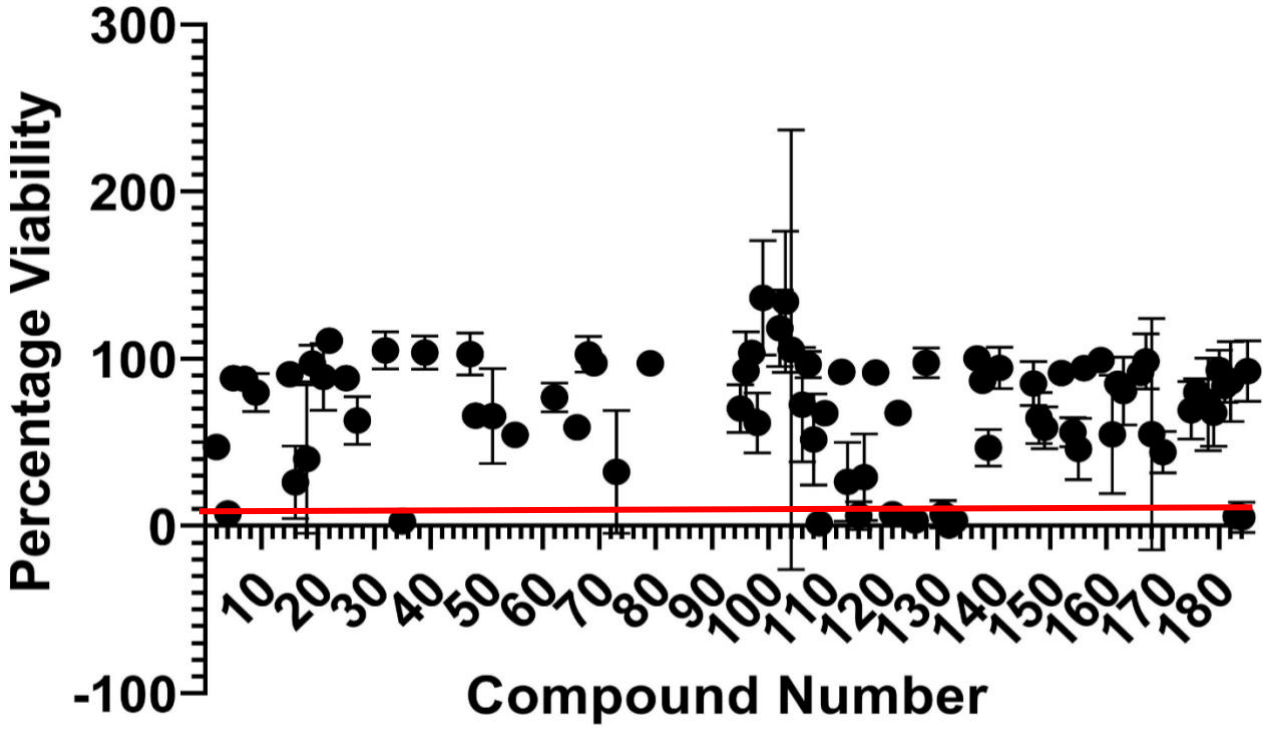


Figure 20 - Secondary screen of the HAT chemical box. *Compounds were tested against T. evansi at a concentration of 0.1µM for 24 hours using the PrestoBlue assay.*

As demonstrated by Figure 21, 2 compounds from the chagas chemical box demonstrated a cell percentage viability of <10% against *T. evansi*. These compounds are - TCMDC-143579, TCMDC-143550.

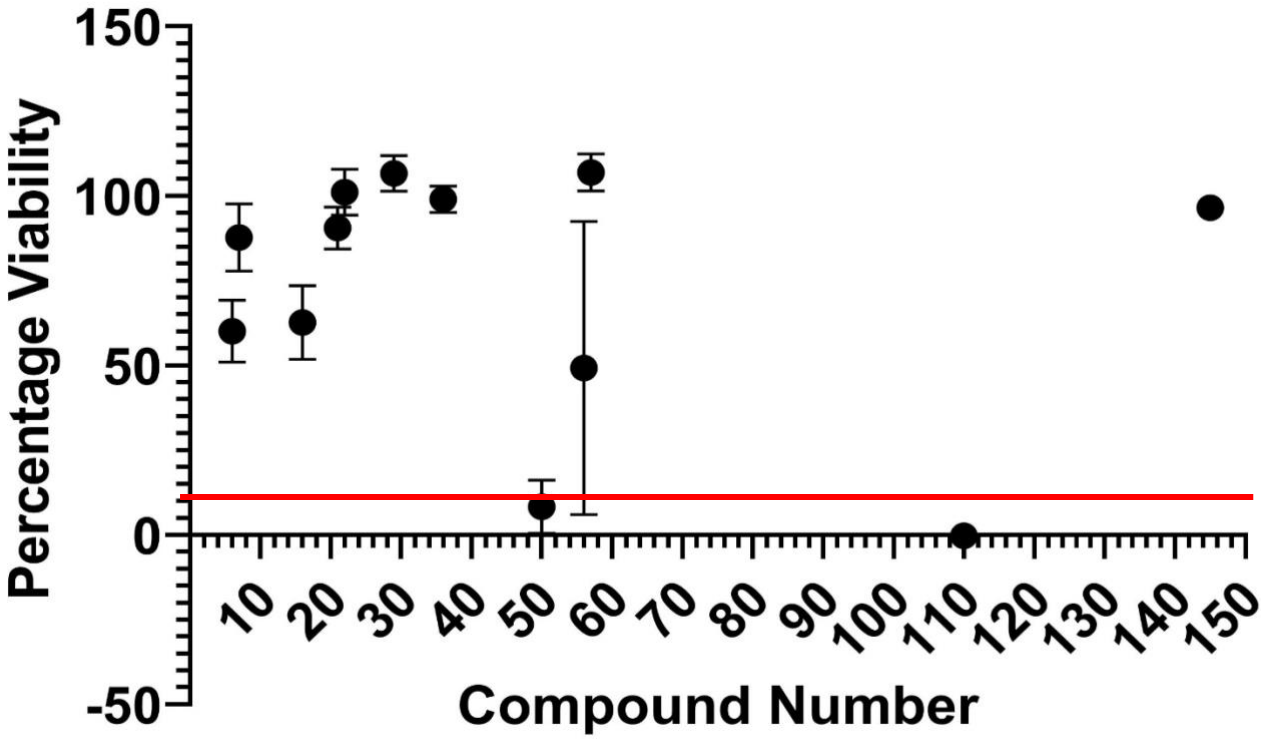


Figure 21 - ***Secondary screen of the HAT chemical box.*** *Compounds were tested against T. evansi at a concentration of 0.1µM for 24 hours using the PrestoBlue assay.*

As demonstrated in Figure 22, no compounds from the leishmaniasis chemical box demonstrated a cell percentage viability of <10% against *T. evansi* at a concentration of 0.1 µM.

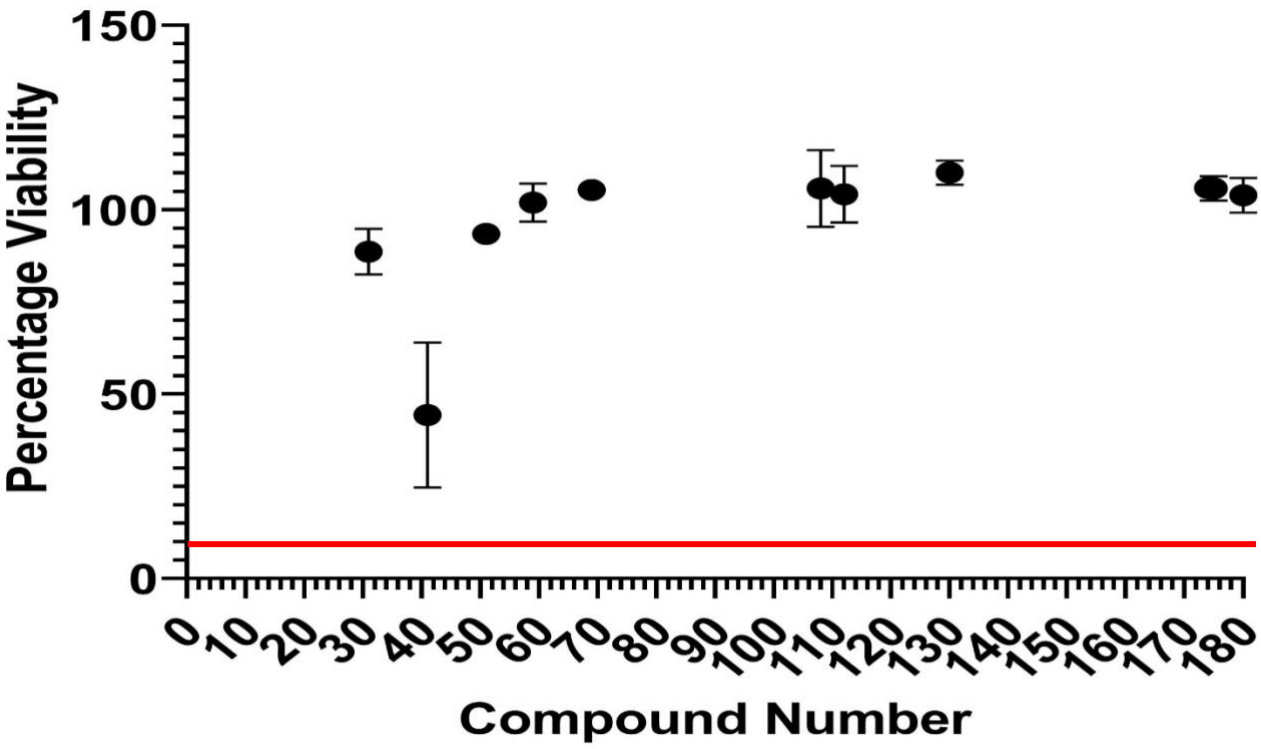


Figure 22 - ***Secondary screen of the HAT chemical box.*** *Compounds were tested against T. evansi at a concentration of 0.1µM for 24 hours using the PrestoBlue assay.*

As seen in the screen at 0.1µM yielded a total of 13 hit compounds. 11 from the HAT box and 2 from the Chagas box. These compounds are demonstrated in Table 4. Like the previous screen, hit compounds were determined as demonstrating less than 10% percentage cell viability in the screen.

3.3.2 EC_{50} of Clomiphene Citrate

A previous study by the Price group demonstrated that clomiphene citrate demonstrated good activity against *T. evansi*. However, the drug showed poor

solubility *in vivo*. This experiment worked to determine the *in vitro* EC50 as shown in Figure 23.

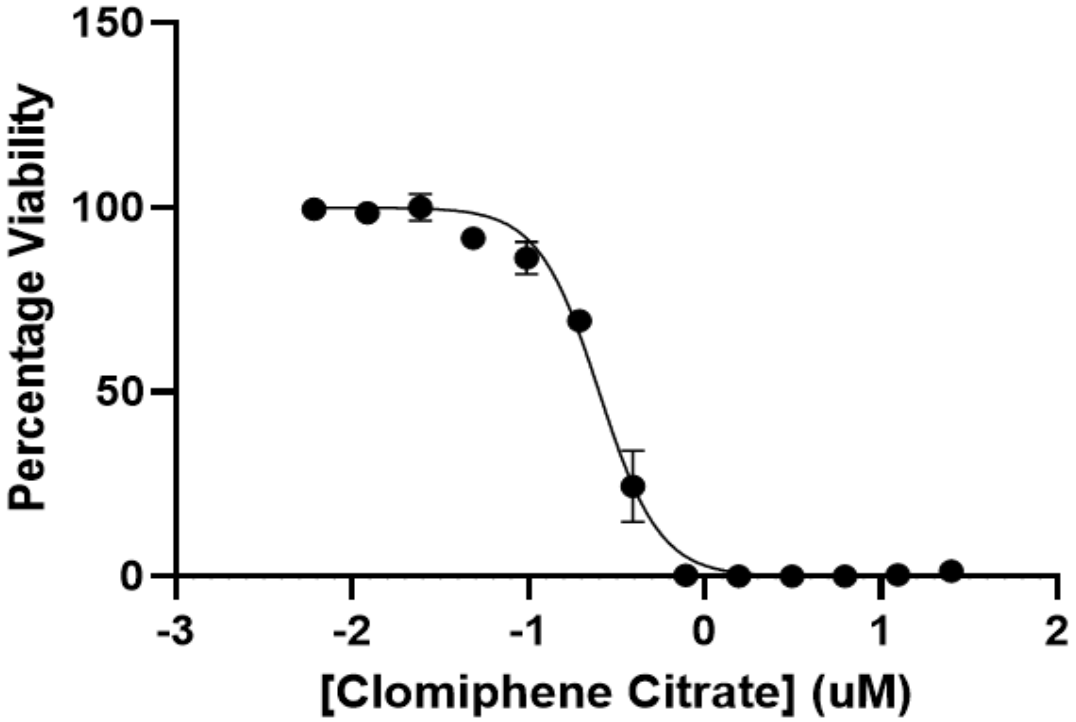


Figure 23 - EC_{50} of clomiphene citrate against T. evansi parasites*. Data points for different concentrations of clomiphene citrate were plotted and the EC_{50} calculated using GraphPad Prism. The average of the three repeats along with standard error of the mean (SEM) bars are shown.*

As demonstrated in Table 4, the calculated value of the EC_{50} of clomiphene citrate was 252nM.

Table 5 - EC_{50} of clomiphene citrate against T. evansi. *The EC_{50} value for clomiphene citrate was identified using the PrestoBlue assay. The EC_{50} value and confidence intervals were calculated using GraphPad Prism*

Drug	EC50 (nM)	95% confidence interval (nM)
Clomiphene Citrate	252	237-268

3.4 Determining the EC_{50} Values of the final 9 hit compounds from the HAT and chagas chemical boxes

The EC_{50} values of 9 hit compounds were determined using the PrestoBlue assay. Table 5 demonstrates the chemical names of all 9 compounds, alongside the EC_{50} value determined in this investigation against *T. evansi* and the EC_{50} value determined in the Peña et al., (2015) against *T. brucei*. As seen in Table 5, the best performing compounds were TCMDC-143326, TCMDC-143341, 143643 with EC_{50} values against *T. evansi* of 10.6 nM, 20.4 nM and 12.0n M respectively. The EC_{50} values of TCMDC-143326 and TCMDC-143341 against *T. evansi* were very close in value to the EC_{50} values against *T. brucei* in the Peña et al., (2015) study, with a <2-fold difference in EC_{50} values between the two species. However, the EC_{50} value of TCMDC-143643 against *T. evansi* is approximately 5 times lower than the EC_{50} value determined against *T. brucei*. TCMDC-143638 demonstrates the biggest EC_{50} value between the two species, with an EC_{50} of 125.9 nM against *T. evansi* and 4.0 nM against *T. brucei*.

Table 6 - The final hit compounds and their EC_{50} values

Compound ID	Chemical name	EC_{50} (*T. evansi*) (nM)	EC_{50} (*T. brucei*) (nM)
TCMDC-143636	N-[4-[(4-pyrazolo[1,5-b]pyridazin-3-ylpyrimidin-2-yl)amino]-2-(trifluoromethyl)phenyl]acetamide	94.5	31.6
TCMDC-143326	2-{4-[1-(1,3-Benzothiazol-2-Yl)-3-Methyl-7-Oxo-4,6,7,8-Tetrahydro-1H-Pyrazolo[3,4-E][1,4]Thiazepin-4-Yl]Phenoxy}Acetamide	10.6	20.0
TCMDC-143251	3-(4-chlorophenyl)-2-methyl-5,6,7,8-tetrahydropyrazolo[5,1-b]quinazolin-9(4H)-one	59.5	158.5
TCMDC-143400	2-[[5-chloro-2-[(2,5-dimethylpyrazol-3-yl)amino]pyrimidin-4-yl]amino]-N-methylbenzenesulfonamide	43.4	63.1
TCMDC-143341	(2S)-1-[4-[(3-Cyanophenyl)methyl]-6-[(5-cyclopropyl-1H-pyrazol-3-yl)amino]pyrimidin-2-yl]azetidine-2-carboxamide	20.4	39.8
TCMDC-143363	N-(5-cyclopropyl-1H-pyrazol-3-yl)-6-methyl-2-(pyrrolidin-1-yl)pyrimidin-4-amine	57.6	$_{50}$.1
TCMDC-143643	2-[3-[(5-bromo-3-methylthiophen-2-yl)methylamino]propylamino]-1H-quinolin-4-one;dihydrochloride	12.0	63.1
TCMDC-143289	3-(4-bromophenyl)-2-methyl-5,6,7,8-tetrahydropyrazolo[5,1-b]quinazolin-9(4H)-one	82.1	398.1
TCMDC-143638	6-[(4-pyrazolo[1,5-b]pyridazin-3-ylpyrimidin-2-yl)amino]-3H-2-benzofuran-1-one.	125.9	4.0

Chapter 4 – Discussion

4.1 Activity of amphotericin B against *T. evansi*

Amphotericin B deoxycholate (DAmB) was introduced as a second line therapy for CL and MCL during the 1960s. Amphotericin B deoxycholate's expensive price and association with renal and systemic toxicity prevented widespread administration of the treatment (Shirzadi, 2019). However, liposomal amphotericin B is a formulation of the drug that is enclosed within a unilamellar liposome. This formulation reduces associated side effects of the drug. The mechanism of action behind both forms of amphotericin B involves the binding to membrane ergosterol. This binding produces an aggregate that causes the cytoplasmic content to leak through a transmembrane channel (Laniado-Laborín and Cabrales-Vargas, 2009). Amphotericin B is not a licensed treatment for *T. evansi* treatment and there is little published data concerning the EC_{50} value for amphotericin B against *T. evansi* parasites *in vitro*.

As seen in Figure 16, amphotericin B had an EC_{50} of 441 nM against *T. evansi.* Da Silva *et al*., (2009) found that concentrations as low as 649 nM demonstrated 100% kill *in vitro* against T. *evansi.* The results of this investigation also agree with a study demonstrating that amphotericin B has an EC_{50} value of approximately 422 nM against blood stream *T. brucei* (Sharma *et al*., 2017). Therefore, amphotericin B was used as the positive control in the PrestoBlue assays in this investigation due to this *in vitro* activity. Although these results show that the drug shows good killing against *T. evansi* cells *in vitro*, the drug is shown to struggle to completely clear parasitaemia *in vivo* (Van Vinh Chau *et al*., 2016;da Silva *et al*. 2007).

4.2 Determining the activity of clomiphene citrate against *T. evansi*

Clomiphene citrate is a racemic mixture made up of *cis* and *trans* isomers. These isomers are named Zuclomiphene and Enclomiphene respectively, with the former

being the more potent of the two isomers (Clark and Markaverich, 1981). The two structures are seen in Figure 24. The mode of action of clomiphene citrate is primarily increasing FSH release from the anterior pituitary leading to ovulation. Clomiphene citrate chemical structure resembles that of estrogen, therefore, blocking hypothalamic estrogen receptors and signalling a lack of circulating estrogen. In turn, this induces an increase in the release of GnRH (Homburg, 2005). There are relatively few reported side effects for the use of clomiphene citrate in humans. Some patients report hot flushes, nausea and mild ovarian enlargement is often common (Homburg, 2005).

As seen in Table 4, clomiphene citrate demonstrated an EC_{50} of 0.252 µM. In comparison the approximate *in vitro* EC_{50} values for Suramin - 0.035 µM (Zoltner *et al*., 2020) and diminazene aceturate – 0.0088 µM (Nyunt *et al*., 2012). Clomiphene citrate is not as potent as some of the currently approved treatments for Surra, however, investigation into other potential advantages over current Surra treatments could prove Clomiphene citrate's potential for use in treatment. For example, Clomiphene citrate demonstrates minimal cytotoxicity against mammals in its common use as a fertility treatment. Demonstrating this as a use for treatment against Surra may provide an advantage over cytotoxic treatments, such as the administration of DA, particularly for canines and camels.

There are numerous reports of clomiphene citrate demonstrating activity against pathogens. Clomiphene citrate can be combined synergistically with colistin to increase antimicrobial activity against planktonic *Psuedomonas aeruginosa* and show antibiofilm activity (Torres *et al*., 2018). There is no data at present on clomiphene citrate demonstrating activity against *T. evansi* or the closely related *T. brucei*. However, using the data gathered on the antimicrobial activity of the drug, we could

infer the possible mode of action against *T. evansi*. Clomiphene also shows activity against *Staphylococcus aureus*. The drug is found to target the enzyme undecaprenyl diphosphate synthase (UPPS) that is involved in the synthesis of polyisoprenoid – an essential part of both peptidoglycan and wall techoic acid synthase (Farha *et al*., 2015). The UPPS enzyme is pivotal to the formation of the cell wall. Although *T. evansi* does not possess the UPPS enzyme, the trypanosome is dependent on the isoprenoid biosynthesis pathways. *T. brucei* do possess farnesyl diphosphate synthase (FPPS) and the enzyme is found to be essential for *T. brucei* cell viability (montalvetti *et al*., 2003). The enzyme catalyses the condensation of dimethylallyl diphosphate (DMAPP) with isopentenyl diphosphate to form geranyl diphosphate (GPP), which in turn, forms farnesyl diphosphate (FPP). Formation of the 15-carbon isoprenoid compound, farnesyl diphosphate is important in *T. brucei* because it acts as a substrate in the enzymatic reactions involved in the biosynthesis of many molecules. These include sterols, ubiquinones, dolichols, heme a and prenylated proteins (Ferella *et al*., 2008).

Durrant *et al*., 2011 discovered in their study of non-bisphosphonate inhibitors of isoprenoid biosynthesis that several predicted inhibitors of FPPS were also inhibitors of UDPS using *T. brucei* FPPS as a model. This is particularly interesting because although the two enzymes are part of the same pathway, they share little sequence or structural homology. At present, there has been no published investigation into the importance of the FPPS enzyme to the survival of *T. evansi*. However, upon comparing the genomic sequence of the *Trypanosoma evansi* STIB 805 FPPS gene and the *Trypanosoma brucei brucei* TREU927 FPPS gene using TriTrypDB, they share 98.9% sequence similarity and a 99.5% predicted protein sequence similarity. This infers that the enzyme is highly likely to have similar functions and substrates as

it does for *T. brucei. T.* evansi FPPS is one of the potential targets of clomiphene citrate.

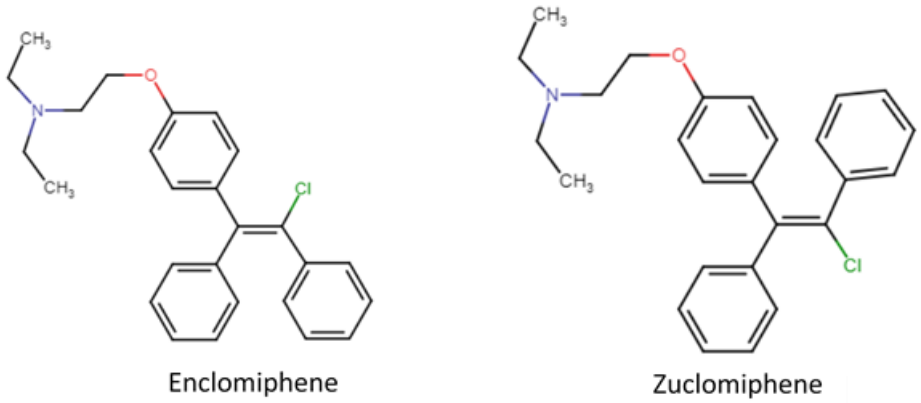


Figure 24** - **The two isomers, enclomiphene and zuclomiphene, that make up the clomiphene racemic mixture.

4.2.1 Clomiphene Citrate and the AAT TPP

If clomiphene citrate were to be considered for treatment of Surra, the drug would have to adhere to the AAT TPP. At this early-stage, clomiphene citrate does adhere to a number of qualities highlighted in the profile. First, there are currently no approved treatments that's mechanism of action involves targeting FPPS, although numerous studies have highlighted that nitrogen-containing bishposphonates, a treatment for bone diseases, do block growth of *T. brucei* parasites by inhibition of FPPS (Muenzker, 2019). If the primary action of clomiphene citrate's primary action was to target FPPS, cross resistance to any current treatments would be limited.

Clomiphene citrate has been tested for reproduction purpose on birds and mammals to a successful effect including cattle and buffalo heifers in India (Zubair and Sajid, 2015;Wankar *et al*., 2017). The treatment also demonstrates good reproductive therapeutic effects in canines (kobayashi *et al*., 2018). The studies do not mention

any adverse side effects of clomiphene citrate on these animals. However, cytotoxicity studies would still be needed to be carried out as increased dosages, modification, or a different method of administration of the drug could be needed to treat *T. evansi* infections.

4.3 Determing the activity of the anti-kinetoplastid compounds against *T.* evansi.

The activity of each anti-kinetoplastid chemical box was tested against *T. evansi* using the PrestoBlue assay. There are a number of examples of open source compound libraries that have been collated to provide a resource for screening against neglected tropical diseases. In 2013, the “Malaria box” was composed made up of 400 chemotypes with half of the compounds selected on their drug-like properties and the other half selected as molecular probes (Spangenberg *et al.,* 2013). In 2011, The National Center for Advancing Translational Sciences (NCATS) developed the Phamaceutical Collection (NPC), a collection of clinically approved drugs. The purpose of this chemical box is the identification of new repurposing candidates for diseases, which is particularly attractive for rare and neglected diseases (Huang *et al.,* 2019).

Compound libraries are excellent examples of scientific collaboration and data sharing. Such libraries allow co-operation between organisations, nonprofit intiatives and academic institutions to more quickly and efficiently address the urgent need for drug development against NTDs (Allarakhia, 2013).

Compound libraries can be target focused which are designed to target individual protein targets or particular protein families (Harris *et al.,* 2011). However, as is the case with the anti-kinetoplastid chemical boxes, compound libraries can have a

diverse selection of targets. GSK used whole-cell phenotypic assays to screen a diverse set of 1.8 million compounds against *L. donovani, T. cruzi* and *T. brucei*. Secondary confirmatory assays were performed on hits and cytotoxicity against human cells was determined. The final compound boxes were constructed after compounds were chemically clustered and triaged for desirable physicochemical properties. The resultant chemical boxes hence have a range of potential modes of action as demonstrated by functional analysis.

The anti-kinetoplastid chemical boxes provide an excellent foundation for successful drug development against Surra. Currently, there is no evidence that this chemical box has been previously tested against *T. evansi*, therefore, identifying compounds with activity against the parasite that have not previously been identified. In addition, as the compound box was tested against the closely related *T. brucei*, a screening could potentially previously unidentified differences between the two species. For example, a compound that demonstrates activity against *T. brucei* but not *T. evansi* may demonstrate differences in the protein target or drug resistance mechanisms between the two species. The discovery of hits against *T. evansi* that are also active against *T. brucei, T. cruzi* and *L. donovani* will also be of great potential benefit. Due to the widespread geographical range of *T. evansi*, there are a number of areas in which these parasites are co-endemic. Identification of compounds with broad spectrum activity against the parasites could be beneficial, however, the development of resistance needs to be considered. Furthermore, cytotoxicity testing of the compounds against specific veterinary cell lines in the future would provide a beneficial addition to the human cell line toxicity assay data already made available.

4.3.1 Screening of the compounds active against HAT

There are several reasons why a portion of the HAT compounds may have fallen short the 10% viability mark in this experiment. Those compounds with an EC_{50} of around 1µM against *T. brucei* would not have progressed to the next screen only 50% of *T. evansi* cells would be killed at this concentration. Another reason why some compounds may not demonstrate the required activity against *T. evansi* cells could be loss of potency and stability due to storage conditions. All compounds were stored at 4°C for several months before use in this screen. Lastly, the differences may be explained by differences in resistance to select compounds due to differences between the two species.

One reason for the differing activity between compounds in the two species could be genetic differences. The most substantial difference between the two bloodstream forms of *T. brucei* and *T. evansi* is the function of the F_0F_1-ATP synthase complex (respiratory complex V) which in eukaryotes, generates ATP through oxidative phosphorylation. In the case of blood stream form *T. brucei* and *T. evansi,* respiratory complex V work in reverse and functions to hydrolyse ATP to pump protons. Both species use this rare mechanism of action to generate an electrochemical potential across the inner mitochondrial membrane (Schnaufer *et al*., 2005). Subunit a of the F_0 section of the complex is kDNA encoded and is critical for proton translocation. However, akinetoplastid *T. evansi* and some laboratory strains of *T. brucei* are capable of surviving without the genes required for this subunit. Dean *et al*. 2013, have demonstrated that a single amino acid change is needed to F_1 subunit γ to allow parasite survival and enables F_0-independent electrochemical potential generation.

Extensive genomic comparisons between the two suggest that the significant phenotypic differences between the two are characterised by subtle genomic changes. Although *T. evansi* can be akinetoplastic, the organism still retains many genes associated with proteins involved in kDNA maintenance, expression and function. (Carnes *et al*., 2015). An example of this is topoisomerase 2, a - an enzyme involved in the replication of kDNA (Balaña-Fouce *et al*., 2014). Although this is the primary role of topoisomerase 2, *T. evansi* cells not expressing this protein show growth defects, indicating another essential role outside of kDNA replication (Dean *et al*., 2013). There are a small number of compounds that target the kinetoplast in their mode of action. Pentamidine, used to treat the acute phase of infection in HAT caused by *T. b. gambiense*, accumulates in the kinetoplast and mitochondria, causing depolorisation of the mitochondrial membrane (Yang *et al*., 2016). Pentamidine also demonstrates efficient killing of *T.* evansi parasites indicating that lethality of the drug is not solely dependent on targeting the kinetoplast (Desquesnes *et al*., 2016). Ethidium bromide, used to treat trypanosome infections in cattle, causes helix distortion of free minicircles causing kDNA loss and cell death. However, ethidium bromide also kills dyskinetoplastic trypanosomes through inhibition of nuclear replication (Chowdhury *et al*., 2010).

In conclusion, although *T. evansi* species have lost kinetoplast genes or are entirely dyskinetoplastic, the species does retain genes that's main functionality is typically associated with the kinetoplast. Therefore, drugs that may target these genes would likely still be effective against *T. evansi* as demonstrated by pentamidine and ethidium bromide. There is little evidence within the literature to suggest that there are any differences in susceptibility between *T. evansi* and *T.* brucei to approved treatments for blood flagellates. As described in Carnes *et al*., (2015), genes involved

in proper function of the kinetoplast may provide other vital functions to the cell. Most lost genes in the evolution of the parasite are related to the oxidative phosphorylation dominant metabolism within the tsetse fly. Although this is important for understanding life cycle and cell morphology, treatments will predominantly be tailored to the bloodstream forms of these trypanosomes, hence, these differences are not as important in treatment investigation.

However, the results highlighted here regarding the difference in activity between both *T. evansi* and *T. brucei* have the capacity to be investigated further. The topic of Surra has little coverage compared to other neglected tropical diseases and there is a possibility that there are further differences between the two species blood stage forms that have yet to be discovered. This could include further experiments to validate that some compounds show decreased activity against *T. evansi,* as well as mode of action studies for the compounds.

4.3.2 Screening of the compounds active against *T. cruzi*

Although *T. evansi* and *T. cruzi* are both from the Trypanosoma genus, the screen indicates that a large majority of the compounds that show excellent activity against *T. cruzi* are not as active against *T. evansi.* The literature shows that it is the difference in interaction between host and parasite that causes the difference in drug effectiveness between *T. cruzi* and *T. evansi*. *T. evansi* is an extracellular parasite and hence is exposed to the host's immune system whilst traveling to secondary infection locations such as major organs and the CNS through the blood (Habila *et al*., 2012). In contrast, *T. cruzi* trypomastigotes take residence inside host cells to migrate to secondary locations as well as changing morphology within cells to the replicative amastigote. These stark differences in life cycle result in a vast difference of genomic differences between *T. evansi* and *T. cruzi* explaining the differences in

susceptibility to the compounds. Such differences in immune evasion and pathogenicity are difficult to target simultaneously, particularly *in vivo*.

There are available treatments that do show effectiveness against both diseases. Nifurtimox is one of the two primary treatments for chagas disease and can also be used in combination with eflornithine to treat stage-2 *gambiense* sleeping sickness (DNDi, 2020). Nifurtimox acts by inducing oxidative stress upon the parasite (Boiani *et al*., 2010). The reduction of a nitro group by the enzyme nitroreductase leads to the generation of a hydroxylamine which can cause damage to the cell directly or aid in the generation of other cytotoxic agents. Artemisinins are also found to inhibit both *T. cruzi* and *T. brucei* growth (Mishina *et al*., 2007).

As 12 compounds in this experiment show activity against T. evansi at concentrations <1 µM, as well as *T. cruzi* in accordance with the GSK HTS, investigation into these compounds further may yield shared targets between the two species.

4.3.3 Compounds active against *Leishmania*

As is the case with *T. cruzi*, *T. evansi* and *Leishmania* species have very different methods of interaction with the host. *Leishmania* promastigotes enter and differentiate into amastigotes in host macrophages before replicating. Cutaneous leishmaniasis causing species are localised in cells and tissue around the area of infection whereas visceral causing species migrate to other organs.

There are common treatment modalities between *T.evansi/T.brucei* and *Leishmania* infection. Pentamidine has been used as a treatment for both, however, pentamidine is only active against the first stage of human African trypanosomiasis, prior to parasites entering the central nervous system (Babokhov *et al*., 2013). As mentioned

previously, pentamidine accumulates in the kinetoplast and mitochondria, causing depolorisation of the mitochondrial membrane (Yang *et al*., 2016). Suramin, a discontinued treatment for Surra, also demonstrates activity against visceral leishmaniasis causing species, and the drug inhibits parasitic phosphoglycerate kinase (Khanra *et al*., 2020).

As 12 compounds in this experiment show activity against T. evansi at concentrations <1 µM, as well as *L. major* in accordance with the GSK HTS, investigation into these compounds further may yield shared targets between the two species.

4.4 The anti-kinetoplastid compounds that demonstrated the best activity against *T. evansi*

As shown in Table 5, 9 compounds were able to demonstrate an EC_{50} <100 nM against *T. evansi*. Several of these compounds share similar motifs, possibly indicating the importance of the motif to activity against *T. evansi*.

4.4.1 2,4 Diaminopyrimidines as potential Surra treatments

One of the common motifs that has emerged during the investigation is the presence of a 2,4 diaminopyrimidine backbone (seen in Figure 25) in several the final compounds that showed the greatest activity against *T. evansi*. These include: TCMDC-143400, TCMDC–143341 and TCMDC-143363. The EC_{50} data and the structure of these compounds can be seen in Table 6.

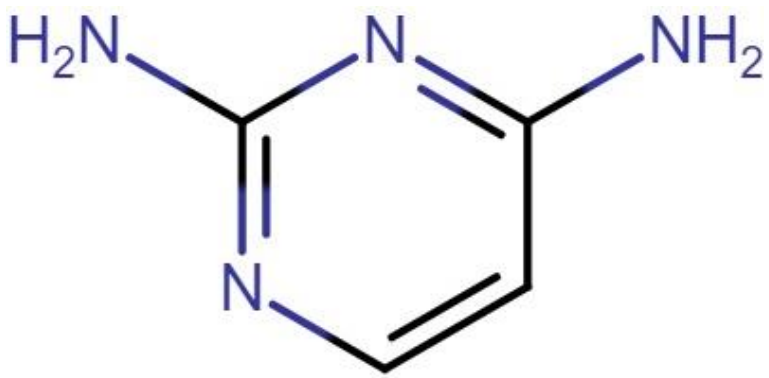


Figure 25 – Typical structure of 2,4 diaminopyrimidine

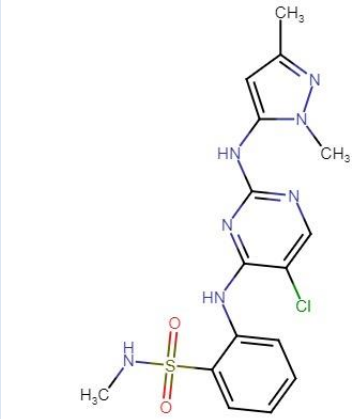
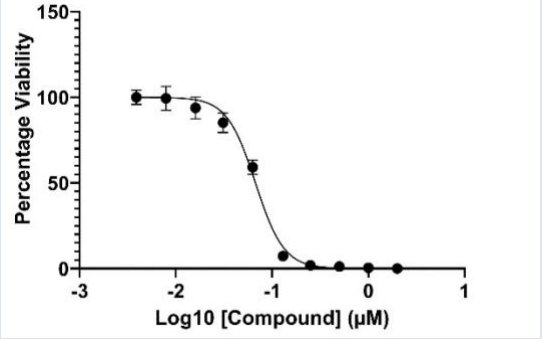
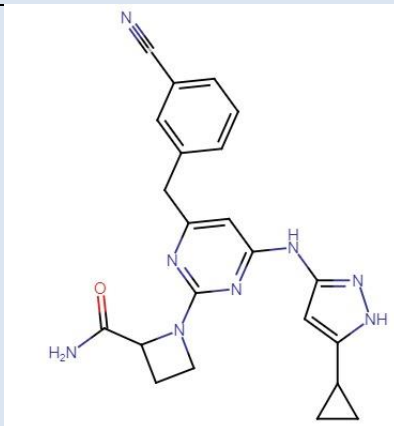
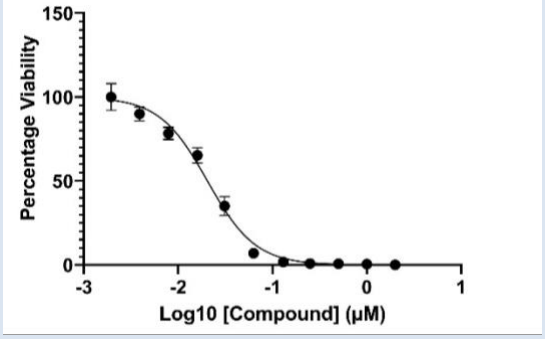
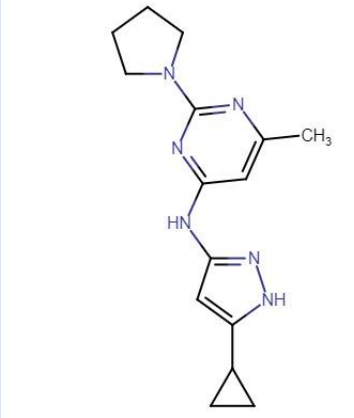
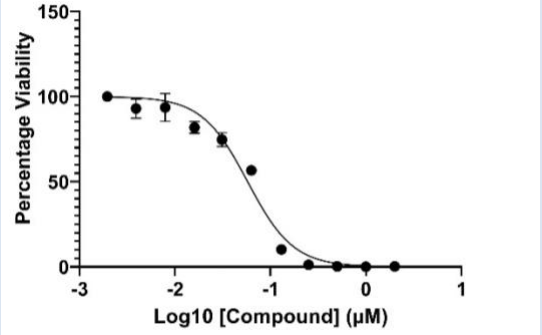
Mercer *et al*., (2011) describe how several 2,4-diaminopyrimidines investigated were active *in vitro* and *in vivo* against all lifecycle stages of *T. brucei* and *L. donovani*. However, this did not include the CNS infecting stage of a *T. brucei* infection. Mercer *et al*., (2011) also indicate that these compounds target *T. brucei* and *L. major* MAPKs and although some 2,4-diaminopyrimidines do target mammalian CDKs, others are selective in their targeting of parasitic MAPKs and/or CRKs. Examining the same anti-kinetoplastid box used in this experiment, Kaiser *et al*., (2015) also found that 2,4-diaminopyrimidine and 2,4-diaminoquinazoline scaffolds were active against *Plasmodium falciparum* (*P. falciparum*), *T. cruzi* and *L. infantum*. However, such compounds show poor permeability through the blood brain barrier and hence, would not be effective against later stages of the disease that have infected the CNS (Perales *et al*., 2011).

TCMDC-143400 contains an N-methylbenzenesulfonamide group that poses a good candidate for the activity of the compound against *T. evansi* activity. The sulfonamide-based class of drugs have shown to have numerous properties including antitumour, antibacterial and anticarbonic anhydrase activity among others (Qadir *et al*., 2015). It is found that compounds having the pyridine and amide functional group commonly exhibit antibacterial and anti-fungal activity. Ugwu *et al*., 2018

synthesised proline derived benzenesulfonamides for use against *T. gambiense*. It was found that these compounds displayed antitrypanosomal and anti-inflammation activities.

TCMDC-143363 was found to demonstrate activity against *Crithidia fasciculate* with a pEC50 of 5.3. Kipandula *et al*. 2018, also highlight that TCMDC-143363 contains a 2-(pyridine-2-yl) pyrimidine-4-amine scaffold, a scaffold that is associated with activity against various kinetoplastids. Colotti *et al*., 2020 demonstrate some compounds possessing this scaffold inhibit trypanothione reductase (TR), an enzyme that aids in the process of defending against reactive oxygen species. TR is an essential enzyme in trypanosomatids. It is important for the catalysing the reduction of trypanothione disulphide to dithiol trypanothione and is essential for proliferation of *Trypanosoma* species. The nearest mammalian homologue of TR is glutathione reductase (GR) which display significant differences in active site (Beig *et al*., 2015).

Table 7 - The structure and EC_{50} values of the 2,4 Diaminopyrimidine based compounds.

Compound ID	Structure	EC_{50} (*T. evansi*) (nM)
TCMDC-143400		 EC_{50} – 67.2 95% CI – 60.9-73.9
TCMDC-143341		 EC_{50} – 20.4 95% CI – 18.3-22.8
TCMDC-143363		 EC_{50} – 57.6 95% CI – 49.4-66.8

4.4.2 Pyrazolo[1,5-b]pyridazines as potential Surra treatments

The Pyrazolo[1,5-b]pyridazine scaffold (demonstrated in Figure 26) was identified in two of the compounds: TCMDC–143638 and TCMDC-143636. Tear *et al*., 2019 identified a number of compounds with Pyrazolo[1,5-b]pyridazine scaffolds that showed activity against *T. b. brucei*. The group showed that compounds with these scaffolds showed selectivity against *T. b. brucei* GSK-3 and CDK over the human homologs, and that these compounds were able to treat mice *in vivo*, as well as demonstrating CNS penetration.

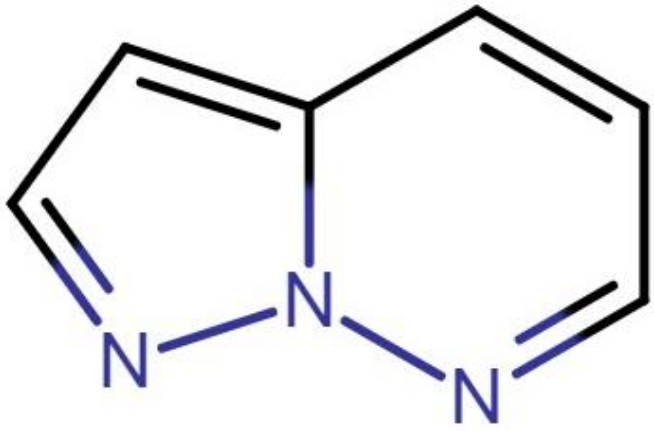
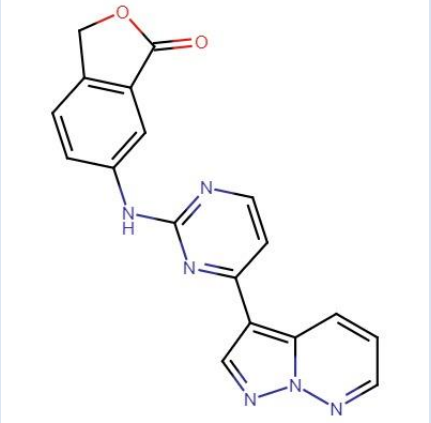
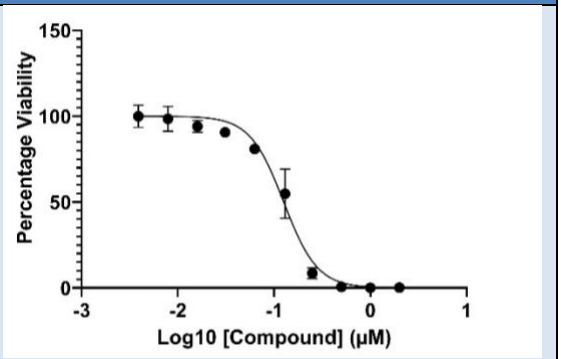
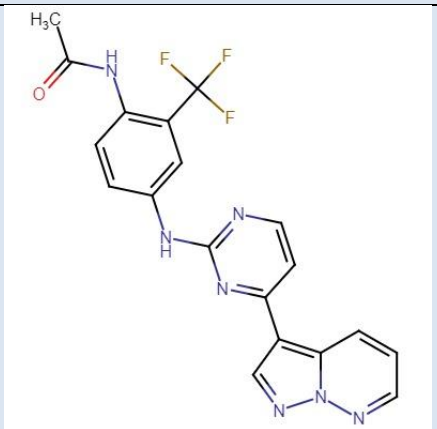
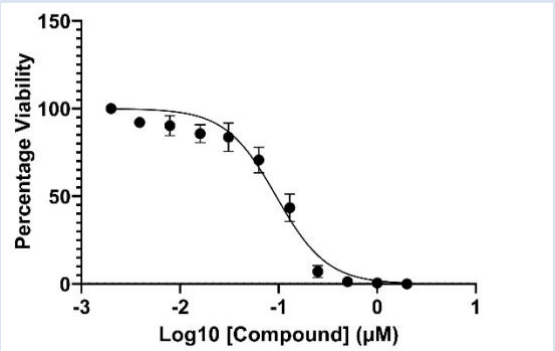


Figure 26** - **The Pyrazolo[1,5-b]pyridazine scaffold

N-Phenyl-4pyrazolo[1,5b]pyridazin-3-ylpyrimidin-2-amines are found to be potent inhibitors of glycogen synthase kinase 3 (GSK-3). GSK-3 is present in all eukaryotes and is a serine/threonine kinase that phosphorylates and downregulates glycogen synthase as well as a multitude of other functions including, but not limited to: cell cycle progression, embryogenesis, apoptosis and cell survival (Juhaszova *et al*., 2009). It is found that interference with the RNA of the two GSK-3 homologues in the bloodstream form of *T. brucei* results in growth arrest and an altered morphology.

Therefore, GSK-3 is required for parasite survival and is a potential drug target (Ojo *et al*., 2008). Ojo *et al*., 2008 also explain that although orthologues exhibit high sequence homology within their catalytic domains, there are a number of differences between human and parasite GSK-3 that could allow the design of parasite specific inhibitors. High throughput screening has identified a number of compounds that act on *Tbru*GSK-3 short with a significant increase in selectivity over the human counterpart *Hs*GSK-3 beta (Odour *et al*., 2011). It is a possibility that TCMDC-143636 could act on *T. evansi* GSK-3 and cause parasite death.

Table 8 - The structure and EC_{50} values of the Pyrazolo[1,5-b]pyridazine based compounds

Compound ID	Structure	EC_{50} (*T. evansi*) (nM)
TCMDC-143638		 EC_{50} – 125.9 95% CI – 113.0-139.5
TCMDC-143636		 EC_{50} – 94.5 95% CI – 77.6-113.9

4.4.3 3-phenylpyrazolo[1-5a]-pyrpyrimidin-7(4H) derivatives as treatments for Surra

TCMDC-143251 and TCMDC-143289 are pyrazoloquinazolines. The compounds are 3-phenylpyrazolo[1-5a]-pyrpyrimidin-7(4H) derivatives with a chemical modification to the initial scaffold as carried out by De Vita *et al*., (2016). The group have shown that TCMDC-143251 and TCMDC-143289 are active against *Myobacterium turberculosis*.

There are 11 different groups of pyrazoloquinazoline, separated based on their ring fusion. These different ring fusions can lead to differing activities of the molecules. TCMDC-143251 and TCMDC-143289 are classified into the [1,5-a] class of pyrazoloquinazolines. This class of compounds are negative allosteric modulators of metabotropic glutamate receptors as well as topoisomerase inhibitors (Garg *et al*., 2015).

Topoisomerases are proteins responsible for introducing breaks in DNA strands, relaxing DNA supercoils during DNA replication or RNA transcription. Topoisomerases can be separated into type 1 and type 2 isomerases. Type 1 isomerases break only one of the DNA strands and are ATP-independent whereas type 2 isomerases break both strands and are ATP-dependent. *T. brucei* also possess topoisomerases with similar functions: Nuclear Top1B and Top2a are involved in DNA replication and repair, mitochondrial Top1A and Top2 are involved with kDNA replication and Top3 resolve DNA entanglements (Balaña-Fouce *et al*, 2014).

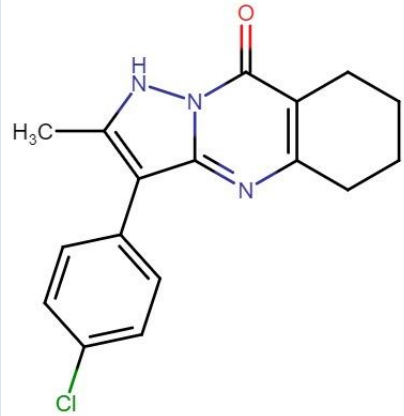
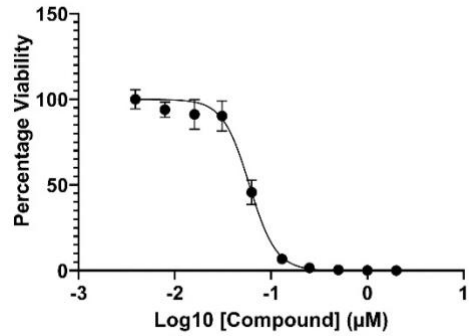
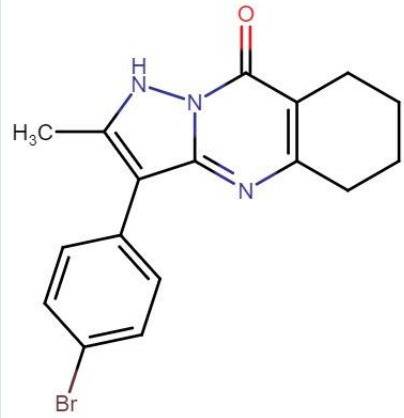
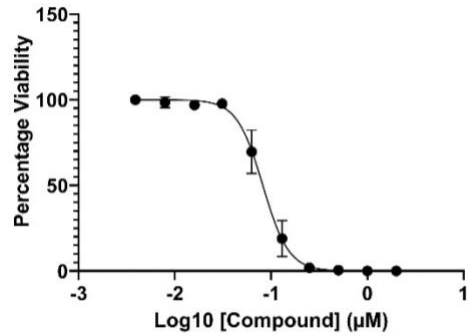
Deterding *et al*. (2005) describe how DNA topoisomerase inhibitors demonstrate excellent anti-trypanosomal activity against bloodstream forms of *T. brucei*. There are several evidenced Top2 inhibitors in trypanosomatids including: fluoroquinolonoes, podophylotoxins, ellipticines, flavonoids, anthracyclins, diamidines, aminocoumarins,

acridines and triterpenoids (Balaña-Fouce *et al*, 2014). As mentioned previously, although topoisomerase 2 is mainly attributed to kDNA activity, targeting of this enzyme also kills *T. evansi*. It is found that a combination of HAT drug eflornithine and top2 inhibitor mitoxantrone not only produced an additive effect but was more selective to trypanosome cells than human cells (Steverding and Wang, 2009).

Although eukaryotic topoisomerases are highly conserved between species, there has been evidence of selectivity for trypanosomes in some inhibitors as mentioned previously (mitoxantrone). The results from the GSK screen indicate that phenylpyrazolo[1-5a]-pyrpyrimidin-7(4H)s also show selectivity towards *T. brucei* however, more investigation against specific species is needed.

As mentioned previously, this class of compounds are also found to be meabotropic glutamate receptors. A literature search as well as TriTrypdb search does not indicate that *T. brucei* possess metabotropic glutamate receptors. Therefore, it is unlikely that TCMDC-143251 and TCMDC-143289 act in this way.

Table 9 - The structure and EC_{50} values of the 3-phenylpyrazolo[1-5a]-pyrpyrimidin-7(4H) based compounds.

Compound ID	Structure	EC_{50} (*T. evansi*) (nM)
TCMDC-143251		 EC_{50} - 59.5 95% CI – 53.2-66.3
TCMDC-143289		 EC_{50} – 82.1 95% CI – 73.4-92.1

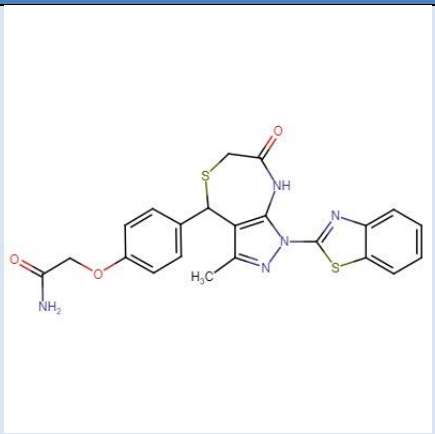
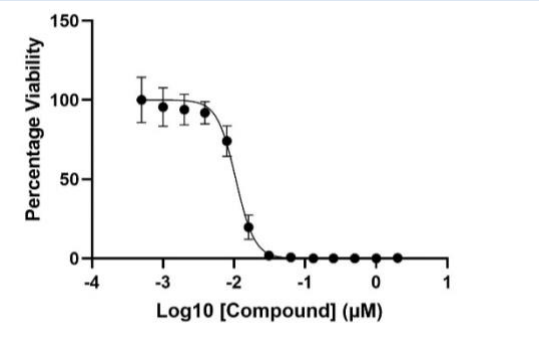
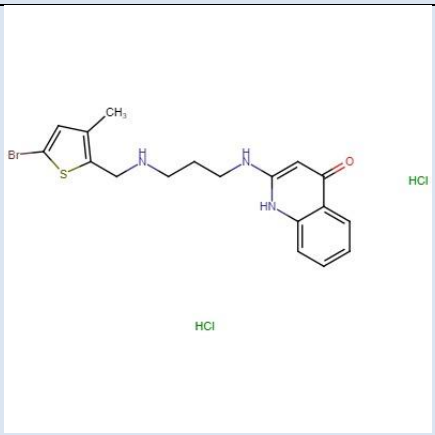
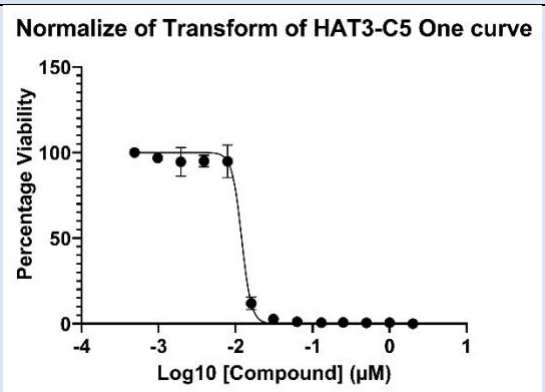
4.4.4 TCMDC-143326 and TCMDC-143643 as treatments for Surra

TCMDC-143326 is a pyrazolo{3,4-e][1,4]thiazepine. Fiuza *et al*., (2018) identify in their study, a number of pyrazolo{3,4-e][1,4]thiazepin based CYP51 inhibitors that show activity against *T. cruzi.* CYP51 is a member of the cytochrome $P4_{50}$ monooxygenase superfamily and mediates the synthesis of ergesterol, a crucial sterol in a number of eukaryotes including trypanosomes (Zhang *et al*., 2019). Uniquely, in *T. brucei's* bloodstream form, it is shown that the parasite incorporates sterols into membranes by taking up low density lipoproteins from its host, into its flagellar pocket (Coppens *et al*., 1988). Nonetheless, CYP51 is found to be essential

in *T. brucei* with reduction of *TbCYP51* expression demonstrating reduced growth with effects being multiple flagella and cytokinesis dysfunction (Dauchy *et al*., 2016). CYP51 inhibitor, posaconazole, is shown to inhibit *T. brucei* growth in mice (Dauchy *et al*., 2016).

TCMDC-143643 is a thiophene compound and such thiophene based analogues have shown to inhibit trypanothione reductase (Patterson *et al*., 2009). As explained previously TryR is found to be essential in the survival of T. brucei.The ChEMBL Target Tree database as part of PubChem indicates that such a compound may target the oxidoreductase class of enzymes. The oxidoreductase class of enzymes are responsible for catalysing oxdidoreduction reactions. These enzymes play an important role in both aerobic and anaerobic metabolism. *T. brucei* possess a number oxidoreductases including a number that are homologous with human equivalents of the protein as well as their own unique oxidoreductase complexes (Panigrahi *et al*., 2008). Oxidoreductases play an important role in the glycolysis dependent metabolism of blood stream form *T. brucei* and *T. evansi* alike and targeting of these enzymes shows good therapeutic potential. The deletion of the gene for the oxidoreductase UDP-Glc-4′-epimerase leads to the death of bloodstream *T. brucei* by halting galactose metabolism (Roper *et al*., 2002)

Table 10 - The structure and EC_{50} values of TCMDC-143326 and TCMDC-143643

Compound ID	Structure	EC_{50} (*T. evansi*)
TCMDC-143326		 EC_{50} – 10.6 95% CI – 9.4-11.9
TCMDC-143643		 EC_{50} – 12.0 95% CI – 11.0-13.0

4.4.5 Compund Adherence to the TPP

Target product profiles (TPPs) provide clear goals and expectations for the drug development process. The AAT TPP provides a number of expectations that can be evaluated in this study.

The first attribute of an ideal treatment for AAT is “A novel agent with new mechanism of action No cross or side resistance to existing product actives.” As demonstrated in section 4.4, the hypothesised mode of action of a number of these groups of compounds differ from current AAT therapeutics

TCMDC-143400, TCMDC–143341 and TCMDC-143363 are 2,4 diaminopyridines ans as discussed previously - are selective in their targeting of parasitic MAPKs and/or CRKs. Currently, there are no treatments for either Surra nor Nagana with this mode of action. TCMDC-143363 contains a 2-(pyridine-2-yl) pyrimidine-4-amine scaffold. Colotti *et al*., 2020 demonstrate some compounds possessing this scaffold inhibit trypanothione reductase (TR), However, the Surra treatment melarsomine also inhibits TR and therefore, further study would be required to investigate cross resistance.

TCMDC–143638 and TCMDC-143636 contain a pyrazolo[1,5-b]pyridazine scaffold. Tear *et al*., 2019 showed that compounds with these scaffolds showed selectivity against *T. b. brucei* GSK-3 and CDK over the human homologs, There is no current treatments for Surra nor Nagana that are suspected to have this mode of action.

TCMDC-143251 and TCMDC-143289 are classified into the [1,5-a] class of pyrazoloquinazolines which are topoisomerase inhibitors (Garg *et al*., 2015). , Isometamidium (treatment of Nagana) and pentamidine (treatment of HAT) are both inhibitors of topoisomerase 2 (Kasozi *et al*., 2022). Isometamidium chloride was a treatment for *T.* evansi infection but is no longer used due to widespread resistance. Therefore, should TCMDC-143251 and TCMDC-143289 have a similar mode of action, they would fail to meet the criteria for this TPP characteristic.

TCMDC-143326 is a pyrazolo{3,4-e][1,4]thiazepine and Fiuza *et al*., (2018) identify in their study, a number of pyrazolo{3,4-e][1,4]thiazepin based CYP51 inhibitors. Should TCMDC-143326 show activity due to inhibition of *T. evansi* this would be a different mechanism of action than that of current Surra or nagana treatments.

Another ideal characteristic for treatment is that the drug must also be able to treat diseases caused by *T. congolense, T. vivax* and *T. brucei* as well as strains resistant to existing trypanocides. As demonstrated in **Table 6**, the compounds demonstrate activity against both *T.* evansi and T*. brucei* (HAT). All compound suggestive targets (Topoisomerase 2, CYP51, GSK-3 and CDK) are common amongst *T. evansi* and the other AATs. However, further investigation is needed to determine activity against *T. congolense, T. vivax* and *T. brucei* as well as resistant strains.

The ideal treatment would be able to be administered to Cattle, sheep, goat + other ruminants, camels, horses, donkeys and pigs. Although this would be have to be tested once the compound has been properly formulated, preliminary cytotoxicity tests could give a basic indication of how such animals may tolerate this compound. Although the GSK HTS demonstrated that the compounds in this study had low cytotoxicity to human HepG2 cells (Peña et al., 2015), further cytotoxicity tests against specific animal cell lines are needed.

Other ideal characteristics include shelf-life as packaged of over 3 years, shelf-life after first opening of more than 7 days and no special precautions required beyond good practice for persons administering the drug. Further investigation would be needed to test the stability of the compounds further, particularly as a final drug formulation. However, this study has proven that each active compound in this study remained stable for 18 months at -4°C.

4.4.6 – Structure-Function analysis of hit compounds

Structure-function analysis is important to evaluate and hypothesise how the drug may perform and interact with other secondary targets when administered *in vivo*. *T. evansi* is a blood parasite that can also infect tissues and later the CNS. Therefore,

consideration needs to be taken on whether active compounds are able to reach these sites.

Pajouhesh and Lenz (2005) defined the ideal characteristics for a successful CNS drug. This includes lipophicility LogP value of 1.5-2.7, a molecular weight <450, for hydrogen bonding a sum of heteroatoms <5, an upper limit of PSA of 90 $Å^2$. Lipinski *et al.,* (1997) developed a rule of 5 which is a general blueprint to a drug having good absorption and permeability:

• Molecular weight is ≤500

• Oil/water distribution coefficient (LogP) is ≤5

• Hydrogen bond donors ≤5 (expressed as the sum of OHs and NHs)

• Hydrogen bond acceptor ≤10 (expressed as the sum of Ns and Os)

• Number of rotatable bonds ≤10

As demonstrated in Table 11, and as seen in the table Table 7 - Table 10 all compounds fit the “rule of 5” indicating good absorption and permeability potential. However, in Paiouhesh and Lenz (2005) characteristics for a successful CNS drug, only TCMDC -143363, TCMDC-143638 and TCMDC-143636 are close to satisfying the criteria to be a successful CNS drug. TCMDC-143363 has a slightly higher value required for LogP (2.8 compared to the adequate range of 1.5-2.7). TCMDC-143638 and TCMDC-143636 both have slightly higher PSA values than the upper limit of 90 with values of 94.3 and 97.1 respectively.

Table 11 - The Molecular weight (MW) oil/water coefficient (LogP) and polar surface area (PSA)

Compound ID	Molecular weight	LogP	PSA
TCMDC-143636	413.65	2.6	97.1
TCMDC-143326	465.56	2.4	112.1
TCMDC-143251	313.79	3.4	50.2
TCMDC-143400	407.884	3.0	113.8
TCMDC-143341	414.74	1.9	136.6
TCMDC-143363	284.367	2.8	69.7
TCMDC-143643	479.27	2.9	56.9
TCMDC-143289	358.241	3.5	50.2
TCMDC-143638	344.35	2.7	94.3

4.4 Summary and Future Work

The 9 compounds that were investigated in the final stages of this project are recorded alongside their corresponding EC_{50} values in Table 5. As seen in the Table 5, all the compounds tested at this stage demonstrated an EC_{50} of approximately 100nM or less against *T. evansi* parasites. As seen by the reference compounds of Suramin and Diminazene aceturate, most compounds show similar potencies to these first line treatments. The next stage of this investigation would include investigating the characteristics of the final hit compounds in more detail.

4.5 Kinetics Investigations

One of the important compound characteristics to consider is the time taken to kill the *T. evansi* cells. Kinetics investigation could be carried out using the PrestoBlue assay as described previously. However, the PrestoBlue assay does not allow for the reading of the results at that particular time point. Although quicker than most resazurin based assays, the PrestoBlue assay is still marketed within an incubation of 10 minutes – 2 hours depending on the cell type. Performing a rate of kill experiment using PrestoBlue would have to account for this "lag" phase of results.

Another method to use in determining compound-mediated killing of *T.* evansi cells would be the CellTiter-Glo reagent. The CellTiter-Glo luminescent cell viability assay determines the number of viable cells based on the quantification of the ATP present which indicates metabolically active cells. The incubation time for the assay is approximately 10 minutes and would allow real time analysis of the killing (Promega, 2021). Bowling *et al*., (2012) used this assay with success investigating the time-kill of a number of compounds in a HTS for HAT treatments.

Genetically modified organisms are an additional way in which rate of kill assays could be performed. Berry *et al*., (2018) describe a transgenic *L. mexicana* cell line that expresses the luciferase NanoLuc-PEST. The transgenic *L. mexicana* was a highly dynamic indicator of cell viability and the results of the bioluminescence-based intramacrophage assay can be read after 3 minutes. Walsh *et al*., (2020) screened a number of compounds using transgenic *T. brucei* that expressed green fluorescent protein (GFP). The results of the screening assay were read immediately using a fluorescence reader. Therefore, genetically modified *T. evansi* could be used to produce immediate results at every time point for a rate of kill assay.

4.6 Cytotoxicity Assays

The GSK HTS demonstrated that the compounds in this study had low cytotoxicity to human HepG2 cells (Peña et al., 2015). However, it would be beneficial to also demonstrate the effects on relevant animal cell lines. As mentioned previously, the therapeutic treatments for Surra can often be extremely cytotoxic to camels, horses and canines. Therefore, testing against cell lines related to these animals would be beneficial. The ATCC have several cell lines available: the camel fibroblast cell line 'Dubca', the Madin Darby Canine Kidney Cell Line and the NZP-36 Equus cell line. The PrestoBlue assay can assess the viability of many different types of eukaryotic cell and therefore, could be used in a cytotoxicity assay to assess the toxic effects of the compounds on the animal cell lines. This would be performed similarly to the compound EC_{50} experiments described previously.

4.7 Mode of Action Investigation

The AAT target product profile recommends the development of a treatment that has a different mode of action to other established treatments for a disease. Therefore, investigation into the mode of action of compounds used in this investigation would be advantageous.

Through the sub-culturing of wild type *T. evansi* cells in the continuous presence of anti-trypanosomal compound, resistant cell lines can be generated. The methodology commonly involves in commencing the culturing in a sub-lethal concentration of the compound and increasing this concentration incrementally. Lima *et al*., (2022) used resistant cell lines to investigate the mode of action of TCMDC-143194 against *T. cruzi*. Whole-genome sequencing of the resistance clones was able to identify single nucleotide polymorphisms in subunits of the proteasome. This method of generating

resistance in trypanosomes has also been demonstrated against nifurtimox and fexinidazole (Wyllie *et al*., 2015) as well as pentamidine (Unciti-Broceta *et al*., 2015). Both the resistant cell line and wild type cell line can be genetically analysed to investigate differences between the two which could potentially highlight the mode of action of the compounds.

Another method of determining the mode of action is the use of proteomics. The effects that compounds can have on cells can be reflected in the proteome of the cell. Often, these changes in the proteome can be determined by liquid chromatography-tandem mass spectrometry and peptide mass fingerprinting (Tulloch *et al*., 2017). Corpas-Lopez and Wyllie, (2021) utilised thermal proteome profiling to identify molecular targets of anti-leishmanial compounds. Drugs that bind to a protein target can significantly alter the thermal stability of the protein and monitoring these changes can identify drug targets. Webster *et al*., (2018) utilised chemical proteomics for analysis of the kinetoplastid folateome – enzymes involved in the folate metabolism of kinetoplastids. A small library of antifolate compounds were immobilised onto resins and used in “pull-down” experiments as demonstrated in Figure 27.

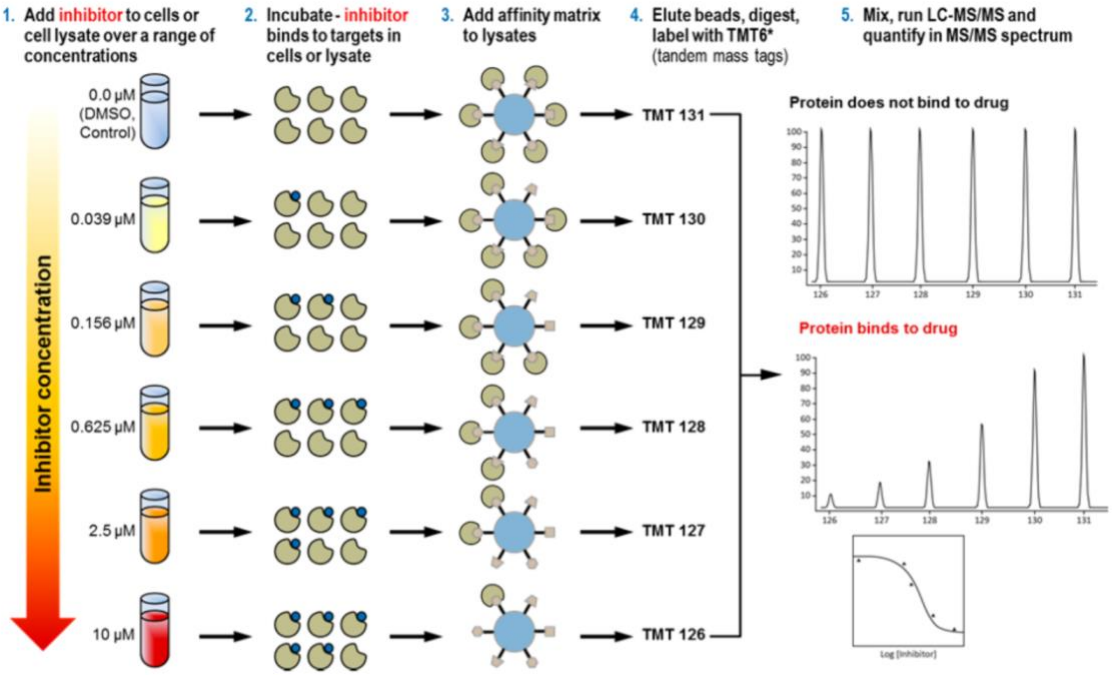


Figure 27 *-* ***Process of identifying foleatome inhibitors through chemical proteomics*** *(Webster et al., 2018)*

4.8 Conclusion

Differences and similarities between kinetoplastids are important factors for treatment research and development. This investigation demonstrates that several compounds that demonstrate activity against *T. brucei* do not demonstrate significant activity against *T. evansi*. These compounds may work on targets that differ between the two species of parasite and determining these mechanisms in future study could highlight more differences between the two. On the other hand, several compounds demonstrated activity against both *L. major* and *T. evansi* as well as *T. cruzi* and *T. evansi,* suggesting there may be common targets between the species.

Novel therapeutic treatments for Surra are desperately needed in endemic areas. This study has further confirmed that fertility drug - clomiphene citrate shows good *in vitro* potential against *T. evansi*. Further investigation into mode of action and increasing its solubility for *in vivo* studies could increase the potential of clomiphene

citrate being considered as a possible treatment for Surra. This investigation also highlighted numerous compounds with excellent activity against *T. evansi* from the GSK anti-kinetoplastid box. Interestingly, a number of these final compounds including TCMDC-143363, TCMDC–143341 and TCMDC–143400 share a 2,4 diaminopyrimidine backbone or a Pyrazolo[1,5-b]pyridazines such as TCMDC–143638 and TCMDC-143636. Other compounds from these two groups could also be investigated for activity against *T. evansi* in the future. Although some of the hit compounds have little to no data available within the literature, others such as TCMDC -143363, TCMDC-143251 and TCMDC-143289 are active against other pathogenic organisms. This gives a potential for a treatment to be used for multiple diseases.

This study has highlighted several compounds that demonstrate activity against *T. evansi* cells. Further study is needed to investigate these compounds further including kinetics investigations, mode of action investigation and cytotoxicity studies on specific livestock cell lines. New drugs for the treatments of Surra would greatly benefit livestock farmers across endemic regions and help to control this important but neglected veterinary disease.

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Appendices

Appendix 1 – HMI-9 media composition

HMI-9 media composition:

- 1.8 g/L HMI-9 Powder (Thermofisher Scientific)
- 0.0036 M NAHCO3 (Thermofisher Scientific)
- 10% HI FBS (Thermofisher Scientific)
- 100U/ml Penicillin (Lonza)
- 100ug/ml Streptomycin (Lonza)
- 4mM L-glutamine (Thermofisher Scientific)
- 5.72mM 2-Mercaptoethanol (Sigma-Aldrich)

Appendix 2 – Raw Data Tables from the first and secondary Anti-kinetoplastid box screening

All compounds used in this experiment were part of the GSK Anti-kinetoplastid compound library. The percentage viability values of all compounds used in both the primary and secondary screens as well as their percentage viability values are demonstrated in the tables below. Alternatively, the full GSK compound database can be found here. The excel version of the raw data from each the primary and secondary compound screen can be found here.

Table 12 - Percentage viability values from the primary screen of the GSK anti-kinetoplastid box (Supplementary data).

HAT Box			**Chagas Box**			**Leish Box**		
TCMDC	**Cell Viability (%)**	**Compound Number (Figure 17)**	**TCMDC**	**Cell Viability (%)**	**Compound Number (Figure 18)**	**TCMDC**	**Cell Viability (%)**	**Compound Number (Figure 19)**
TCMDC-143234	83.80	1	TCMDC-143203	127.06	1	TCMDC-143072	130.00	1
TCMDC-143526	9.28	2	TCMDC-143200	133.10	2	TCMDC-143629	83.20	2
TCMDC-143130	19.20	3	TCMDC-143235	128.41	3	TCMDC-143443	117.00	3
TCMDC-143644	2.02	4	TCMDC-143411	113.64	4	TCMDC-143217	114.00	4

TCMDC-143569	6.14	5	TCMDC-143232	144.51	5	TCMDC-143459	138.00	5
TCMDC-143466	124.00	6	TCMDC-143612	0.98	6	TCMDC-143480	123.00	6
TCMDC-143446	9.68	7	TCMDC-143127	0.04	7	TCMDC-143501	128.00	7
TCMDC-143273	135.00	8	TCMDC-143588	130.42	8	TCMDC-143196	131.00	8
TCMDC-143299	5.51	9	TCMDC-143109	139.81	9	TCMDC-143260	138.00	9
TCMDC-143544	56.90	10	TCMDC-143153	133.77	10	TCMDC-124508	123.00	10
TCMDC-143270	119.00	11	TCMDC-143149	147.86	11	TCMDC-143512	127.00	11
TCMDC-143368	113.00	12	TCMDC-143191	108.95	12	TCMDC-134026	134.00	12
TCMDC-143366	10.90	13	TCMDC-143300	152.56	13	TCMDC-143487	124.00	13
TCMDC-143596	44.30	14	TCMDC-143319	137.80	14	TCMDC-143621	126.00	14
TCMDC-143370	3.34	15	TCMDC-143598	133.10	15	TCMDC-143442	126.00	15
TCMDC-143475	6.14	16	TCMDC-143308	-0.09	16	TCMDC-125826	121.00	16
TCMDC-143444	14.00	17	TCMDC-143610	141.83	17	TCMDC-143630	122.00	17
TCMDC-143424	7.97	18	TCMDC-143405	143.17	18	TCMDC-143215	138.00	18
TCMDC-143642	6.54	19	TCMDC-143484	98.21	19	TCMDC-143180	127.00	19
TCMDC-143079	136.00	20	TCMDC-143467	132.43	20	TCMDC-143211	131.00	20
TCMDC-143609	3.39	21	TCMDC-143417	6.96	21	TCMDC-143078	109.00	21

TCMDC-143635	0.02	22	TCMDC-143381	4.88	22	TCMDC-143216	119.00	22
TCMDC-143080	112.00	23	TCMDC-143613	133.10	23	TCMDC-143212	147.00	23
TCMDC-143470	95.80	24	TCMDC-143548	140.48	24	TCMDC-143213	115.00	24
TCMDC-143636	9.81	25	TCMDC-143190	147.19	25	TCMDC-143077	108.00	25
TCMDC-143163	124.00	26	TCMDC-143209	141.15	26	TCMDC-143076	128.00	26
TCMDC-143360	-13.28	27	TCMDC-143311	141.83	27	TCMDC-143197	107.00	27
TCMDC-143438	14.00	28	TCMDC-143387	54.60	28	TCMDC-143315	119.00	28
TCMDC-143194	20.92	29	TCMDC-143298	5.35	29	TCMDC-143306	117.00	29
TCMDC-143425	15.73	30	TCMDC-143615	145.85	30	TCMDC-143305	129.00	30
TCMDC-143525	31.31	31	TCMDC-143163	143.84	31	TCMDC-143236	7.54	31
TCMDC-142716	7.50	32	TCMDC-143164	135.79	32	TCMDC-143327	127.00	32
TCMDC-143533	14.00	33	TCMDC-143197	92.17	33	TCMDC-143348	129.00	33
TCMDC-143515	72.01	34	TCMDC-143315	125.05	34	TCMDC-143345	116.00	34
TCMDC-143579	-0.07	35	TCMDC-143497	142.50	35	TCMDC-143355	85.30	35
TCMDC-143587	42.35	36	TCMDC-143526	9.91	36	TCMDC-143340	147.00	36
TCMDC-143578	176.98	37	TCMDC-143130	155.24	37	TCMDC-143353	141.00	37
TCMDC-143624	65.73	38	TCMDC-143273	143.84	38	TCMDC-143344	131.00	38

TCMDC-143648	2.74	39	TCMDC-143569	17.69	39	TCMDC-143358	136.00	39
TCMDC-143121	26.12	40	TCMDC-143466	141.83	40	TCMDC-143383	151.00	40
TCMDC-143583	15.30	41	TCMDC-143446	144.51	41	TCMDC-143350	6.15	41
TCMDC-143138	21.36	42	TCMDC-143082	130.42	42	TCMDC-143388	140.00	42
TCMDC-143131	67.68	43	TCMDC-143194	131.09	43	TCMDC-143075	83.60	43
TCMDC-143364	43.22	44	TCMDC-143465	151.22	44	TCMDC-143249	137.00	44
TCMDC-143399	12.91	45	TCMDC-143080	145.85	45	TCMDC-143246	131.00	45
TCMDC-143402	207.29	46	TCMDC-143413	134.44	46	TCMDC-143281	130.00	46
TCMDC-143436	3.17	47	TCMDC-143422	135.79	47	TCMDC-143280	129.00	47
TCMDC-143393	4.69	48	TCMDC-143385	145.18	48	TCMDC-143404	113.00	48
TCMDC-143158	196.46	49	TCMDC-143083	37.15	49	TCMDC-143347	129.00	49
TCMDC-145401	18.76	50	TCMDC-143550	1.86	50	TCMDC-143351	166.00	50
TCMDC-143493	7.29	51	TCMDC-143494	139.81	51	TCMDC-143375	8.99	51
TCMDC-143496	267.89	52	TCMDC-143085	143.17	52	TCMDC-143398	139.00	52
TCMDC-143499	267.89	53	TCMDC-143561	130.42	53	TCMDC-143086	144.00	53
TCMDC-143240	425.90	54	TCMDC-143490	133.10	54	TCMDC-143396	133.00	54
TCMDC-143516	6.20	55	TCMDC-143421	133.77	55	TCMDC-143524	131.00	55

TCMDC-143589	19.19	56	TCMDC-143500	2.80	56	TCMDC-143628	169.00	56
TCMDC-143547	55.99	57	TCMDC-143423	7.02	57	TCMDC-143603	121.00	57
TCMDC-143089	14.00	58	TCMDC-139489	133.77	58	TCMDC-143647	142.00	58
TCMDC-143581	37.16	59	TCMDC-143502	127.06	59	TCMDC-143618	0.37	59
TCMDC-143575	246.25	60	TCMDC-143150	72.20	60	TCMDC-143397	132.00	60
TCMDC-143134	15.95	61	TCMDC-143178	123.52	61	TCMDC-143451	136.00	61
TCMDC-143173	5.56	62	TCMDC-143143	155.60	62	TCMDC-143297	144.00	62
TCMDC-143167	11.40	63	TCMDC-143187	159.87	63	TCMDC-143391	54.70	63
TCMDC-143116	244.08	64	TCMDC-143331	188.74	64	TCMDC-143349	115.00	64
TCMDC-143195	21.14	65	TCMDC-143317	153.46	65	TCMDC-143367	137.00	65
TCMDC-143189	6.42	66	TCMDC-143338	183.39	66	TCMDC-143478	19.40	66
TCMDC-143228	10.10	67	TCMDC-143604	174.84	67	TCMDC-143536	133.00	67
TCMDC-143226	4.04	68	TCMDC-143408	164.15	68	TCMDC-143483	121.00	68
TCMDC-143192	8.15	69	TCMDC-143409	131.00	69	TCMDC-143482	1.23	69
TCMDC-143303	15.08	70	TCMDC-143415	162.22	70	TCMDC-143274	161.00	70
TCMDC-143267	81.75	71	TCMDC-143426	80.76	71	TCMDC-143574	122.00	71
TCMDC-143456	138.02	72	TCMDC-143207	167.36	72	TCMDC-143554	135.00	72

TCMDC-143505	7.72	73	TCMDC-143530	214.40	73	TCMDC-143573	136.00	73
TCMDC-143342	42.78	74	TCMDC-143507	148.11	74	TCMDC-143633	124.00	74
TCMDC-143641	11.18	75	TCMDC-143593	246.47	75	TCMDC-143296	132.00	75
TCMDC-143543	287.37	76	TCMDC-143088	212.26	76	TCMDC-143238	103.00	76
TCMDC-143551	114.21	77	TCMDC-143142	267.85	77	TCMDC-143099	75.50	77
TCMDC-143449	90.40	78	TCMDC-143161	193.01	78	TCMDC-143285	113.00	78
TCMDC-143556	4.91	79	TCMDC-143155	267.85	79	TCMDC-143491	61.60	79
TCMDC-143565	144.52	80	TCMDC-143222	243.26	80	TCMDC-143607	135.00	80
TCMDC-143572	168.33	81	TCMDC-143276	180.19	81	TCMDC-143521	126.00	81
TCMDC-143595	300.36	82	TCMDC-143626	263.58	82	TCMDC-143486	138.00	82
TCMDC-143100	59.45	83	TCMDC-143372	145.97	83	TCMDC-143427	142.00	83
TCMDC-143104	18.54	84	TCMDC-143601	246.47	84	TCMDC-143429	117.00	84
TCMDC-143107	47.11	85	TCMDC-143617	216.54	85	TCMDC-143503	20.00	85
TCMDC-143540	16.16	86	TCMDC-143537	259.30	86	TCMDC-143431	136.00	86
TCMDC-143132	235.43	87	TCMDC-143279	203.71	87	TCMDC-143419	128.00	87
TCMDC-143230	18.98	88	TCMDC-143084	260.37	88	TCMDC-143418	121.00	88
TCMDC-143229	12.27	89	TCMDC-143620	296.72	89	TCMDC-143509	131.00	89

TCMDC-143231	57.50	90	TCMDC-143362	259.30	90	TCMDC-143473	129.00	90
TCMDC-143312	24.60	91	TCMDC-143314	262.51	91	TCMDC-143488	137.00	91
TCMDC-143374	11.18	92	TCMDC-143414	253.96	92	TCMDC-143568	127.00	92
TCMDC-143320	71.14	93	TCMDC-123621	227.23	93	TCMDC-143406	114.00	93
TCMDC-143452	15.30	94	TCMDC-143552	244.33	94	TCMDC-143295	104.00	94
TCMDC-143453	2.74	95	TCMDC-143555	164.15	95	TCMDC-143278	123.00	95
TCMDC-143640	9.24	96	TCMDC-143097	166.29	96	TCMDC-143136	107.00	96
TCMDC-143645	-10.03	97	TCMDC-143256	304.21	97	TCMDC-143407	115.00	97
TCMDC-143394	6.42	98	TCMDC-143253	78.62	98	TCMDC-143269	122.00	98
TCMDC-143073	-2.67	99	TCMDC-143262	315.97	99	TCMDC-143447	126.00	99
TCMDC-143585	159.67	100	TCMDC-143611	233.64	100	TCMDC-142900	146.00	100
TCMDC-143111	21.36	101	TCMDC-143520	260.37	101	TCMDC-143163	114.00	101
TCMDC-143125	-4.40	102	TCMDC-143602	281.75	102	TCMDC-143164	117.00	102
TCMDC-143146	7.72	103	TCMDC-143606	207.98	103	TCMDC-143113	123.00	103
TCMDC-143225	3.39	104	TCMDC-143637	199.43	104	TCMDC-143133	98.30	104
TCMDC-143205	12.48	105	TCMDC-143464	220.81	105	TCMDC-143448	83.30	105
TCMDC-143210	7.50	106	TCMDC-143614	229.37	106	TCMDC-143115	101.00	106

TCMDC-143199	-6.69	107	TCMDC-143625	421.81	107	TCMDC-143639	103.00	107
TCMDC-143251	1.82	108	TCMDC-143535	291.38	108	TCMDC-143145	9.60	108
TCMDC-143250	-3.64	109	TCMDC-143632	327.73	109	TCMDC-143508	13.80	109
TCMDC-143219	-2.16	110	TCMDC-143527	1.85	110	TCMDC-143517	100.00	110
TCMDC-143243	51.90	111	TCMDC-143186	287.10	111	TCMDC-143531	106.00	111
TCMDC-143220	90.80	112	TCMDC-143224	19.60	112	TCMDC-143522	-2.13	112
TCMDC-143263	-0.31	113	TCMDC-143248	280.68	113	TCMDC-143534	97.70	113
TCMDC-143307	-5.39	114	TCMDC-143284	139.56	114	TCMDC-143532	117.00	114
TCMDC-143330	12.30	115	TCMDC-143286	283.89	115	TCMDC-143523	14.40	115
TCMDC-143373	-7.52	116	TCMDC-143309	259.30	116	TCMDC-143518	102.00	116
TCMDC-143363	-2.16	117	TCMDC-143631	231.50	117	TCMDC-143570	109.00	117
TCMDC-143369	14.90	118	TCMDC-143376	242.20	118	TCMDC-143577	107.00	118
TCMDC-143386	2.00	119	TCMDC-143371	260.37	119	TCMDC-143563	114.00	119
TCMDC-143352	14.30	120	TCMDC-143410	274.27	120	TCMDC-143567	90.80	120
TCMDC-143458	16.70	121	TCMDC-143403	229.37	121	TCMDC-143586	117.00	121
TCMDC-143454	1.17	122	TCMDC-143455	66.86	122	TCMDC-143584	89.10	122
TCMDC-143401	0.99	123	TCMDC-143599	106.41	123	TCMDC-143591	106.00	123

TCMDC-143382	179.00	124	TCMDC-143479	206.91	124	TCMDC-143594	112.00	124
TCMDC-143154	243.00	125	TCMDC-143440	193.01	125	TCMDC-143576	107.00	125
TCMDC-143206	-0.12	126	TCMDC-143592	235.78	126	TCMDC-143538	101.00	126
TCMDC-143227	13.60	127	TCMDC-143105	182.32	127	TCMDC-143514	108.00	127
TCMDC-143233	1.45	128	TCMDC-143120	297.79	128	TCMDC-143093	113.00	128
TCMDC-143264	171.00	129	TCMDC-143160	134.21	129	TCMDC-143090	109.00	129
TCMDC-143294	88.90	130	TCMDC-143310	232.57	130	TCMDC-143091	4.71	130
TCMDC-143326	2.47	131	TCMDC-143325	203.71	131	TCMDC-143092	126.00	131
TCMDC-143335	2.00	132	TCMDC-125222	34.40	132	TCMDC-143095	119.00	132
TCMDC-143337	6.53	133	TCMDC-143519	10.50	133	TCMDC-143094	105.00	133
TCMDC-143361	44.50	134	TCMDC-143546	162.00	134	TCMDC-143096	112.00	134
TCMDC-143510	248.00	135	TCMDC-143562	112.00	135	TCMDC-143098	103.00	135
TCMDC-143634	22.20	136	TCMDC-143549	14.70	136	TCMDC-143566	105.00	136
TCMDC-143365	3.30	137	TCMDC-143151	80.60	137	TCMDC-143117	106.00	137
TCMDC-143380	4.13	138	TCMDC-143185	138.00	138	TCMDC-143106	114.00	138
TCMDC-143074	6.07	139	TCMDC-143193	140.00	139	TCMDC-143110	109.00	139
TCMDC-143582	130.00	140	TCMDC-143241	150.00	140	TCMDC-143119	111.00	140

TCMDC-143643	0.15	141	TCMDC-143247	151.00	141	TCMDC-143144	115.00	141
TCMDC-143123	76.00	142	TCMDC-143332	202.00	142	TCMDC-142704	114.00	142
TCMDC-143457	10.30	143	TCMDC-143071	215.00	143	TCMDC-143101	113.00	143
TCMDC-143462	47.30	144	TCMDC-143346	224.00	144	TCMDC-125387	93.70	144
TCMDC-143428	26.00	145	TCMDC-143334	2.56	145	TCMDC-143124	82.20	145
TCMDC-143445	117.00	146	TCMDC-143333	245.00	146	TCMDC-143140	108.00	146
TCMDC-143254	1.54	147	TCMDC-143354	247.00	147	TCMDC-143141	120.00	147
TCMDC-142497	9.49	148	TCMDC-143182	225.00	148	TCMDC-143571	116.00	148
TCMDC-143638	2.00	149	TCMDC-143384	219.00	149	TCMDC-143184	89.70	149
TCMDC-143513	15.30	150	TCMDC-143389	97.20	150	TCMDC-143129	115.00	150
TCMDC-143597	290.00	151	TCMDC-143461	216.00	151	TCMDC-143147	111.00	151
TCMDC-143580	6.53	152	TCMDC-143379	201.00	152	TCMDC-143139	151.00	152
TCMDC-143128	27.00	153	TCMDC-143416	262.00	153	TCMDC-143174	115.00	153
TCMDC-143172	8.38	154	TCMDC-143477	263.00	154	TCMDC-143169	94.30	154
TCMDC-143357	3.20	155	TCMDC-143430	68.60	155	TCMDC-143171	14.40	155
TCMDC-143400	4.68	156	TCMDC-143476	181.00	156	TCMDC-143168	109.00	156
TCMDC-143435	14.00	157	TCMDC-143152	202.00	157	TCMDC-143175	116.00	157

TCMDC-143257	14.20	158	TCMDC-143432	68.60	158	TCMDC-143165	125.00	158
TCMDC-143460	2.93	159	TCMDC-143542	173.00	159	TCMDC-143166	140.00	159
TCMDC-143469	11.50	160	TCMDC-143126	249.00	160	TCMDC-143188	130.00	160
TCMDC-143390	0.06	161	TCMDC-143282	171.00	161	TCMDC-143202	120.00	161
TCMDC-143468	2.65	162	TCMDC-143258	240.00	162	TCMDC-143214	115.00	162
TCMDC-143471	0.80	163	TCMDC-143275	223.00	163	TCMDC-125160	87.40	163
TCMDC-143102	303.00	164	TCMDC-143288	253.00	164	TCMDC-143558	70.10	164
TCMDC-143619	31.60	165	TCMDC-143081	250.00	165	TCMDC-143557	24.10	165
TCMDC-143112	9.12	166	TCMDC-143474	222.00	166	TCMDC-143237	18.40	166
TCMDC-143242	-1.69	167	TCMDC-143293	201.00	167	TCMDC-143218	111.00	167
TCMDC-143292	2.56	168	TCMDC-143118	178.00	168	TCMDC-143239	108.00	168
TCMDC-143265	63.00	169	TCMDC-143103	180.00	169	TCMDC-143181	126.00	169
TCMDC-143289	-0.03	170	TCMDC-143137	245.00	170	TCMDC-143201	98.30	170
TCMDC-143283	168.00	171	TCMDC-143291	245.00	171	TCMDC-143208	125.00	171
TCMDC-143318	246.00	172	TCMDC-143162	253.00	172	TCMDC-143271	120.00	172
TCMDC-143316	21.30	173	TCMDC-143564	258.00	173	TCMDC-143245	122.00	173
TCMDC-143646	191.00	174	TCMDC-143313	225.00	174	TCMDC-143277	8.16	174

TCMDC-143359	1.36	175	TCMDC-143328	245.00	175	TCMDC-143223	3.56	175
TCMDC-143176	0.99	176	TCMDC-143336	290.00	176	TCMDC-143170	12.10	176
TCMDC-143204	30.70	177	TCMDC-140766	197.00	177	TCMDC-143252	107.00	177
TCMDC-143378	2.37	178	TCMDC-143504	210.00	178	TCMDC-143255	97.70	178
TCMDC-143343	-3.64	179	TCMDC-143272	135.00	179	TCMDC-143287	93.70	179
TCMDC-143341	2.93	180	TCMDC-143605	241.00	180	TCMDC-143261	3.28	180
TCMDC-143356	-0.59	181	TCMDC-143506	278.00	181	TCMDC-143268	115.00	181
TCMDC-143412	-5.21	182	TCMDC-143437	282.00	182	TCMDC-143259	109.00	182
TCMDC-143377	-2.43	183	TCMDC-143244	229.00	183	TCMDC-143266	110.00	183
TCMDC-143339	-1.51	184	TCMDC-143539	245.00	184			
TCMDC-143392	-11.30	185	TCMDC-143553	229.00	185			
TCMDC-143290	0.15	186	TCMDC-143329	195.00	186			
			TCMDC-143087	94.50	187			
			TCMDC-143511	180.00	188			
			TCMDC-143559	238.00	189			
			TCMDC-143324	253.00	190			
			TCMDC-143622	250.00	191			

			TCMDC-143492	269.00	192			
			TCMDC-143108	248.00	193			
			TCMDC-143135	216.00	194			
			TCMDC-143304	75.00	195			
			TCMDC-143495	162.00	196			
			TCMDC-143179	143.00	197			
			TCMDC-143481	148.00	198			
			TCMDC-143623	121.00	199			
			TCMDC-143159	191.00	200			
			TCMDC-143148	198.00	201			
			TCMDC-143301	187.00	202			
			TCMDC-143302	204.00	203			

Table 13 - Percentage viability values from the Secondary screen of the GSK anti-kinetoplastid box (Supplementary data).

HAT Box			Chagas Box			Leishmania Box		
TCMDC	**Percentage Viability**	**Compound Number (Figure 20)**	**TCMDC**	**Percentage Viability**	**Compound Number (Figure 21)**	**TCMDC**	**Percentage Viability**	**Compound Number (Figure 22)**
TCMDC-143299	47.22	2	TCMDC-143612	60.07	6	TCMDC-143618	88.59	31
TCMDC -143636	7.43	4	TCMDC-143127	87.70	7	TCMDC-143236	44.32	41
TCMDC -143526	88.45	5	TCMDC-143308	62.61	16	TCMDC-143350	93.43	51
TCMDC-143424	88.01	7	TCMDC-143417	90.48	21	TCMDC-143375	101.93	59
TCMDC-143644	79.87	9	TCMDC-143381	101.08	22	TCMDC-143482	105.32	69
TCMDC-143569	90.70	15	TCMDC-143526	106.60	29	TCMDC-143145	105.76	108
TCMDC-143475	26.16	16	TCMDC-143298	98.98	36	TCMDC-143522	104.17	112
TCMDC-143642	40.15	18	TCMDC-143550	8.27	50	TCMDC-143091	110.03	130
TCMDC-143609	97.21	19	TCMDC-143500	49.18	56	TCMDC-143277	105.86	174
TCMDC-143446	89.18	21	TCMDC-143423	106.84	57	TCMDC-143223	105.72	175
TCMDC-	110.86	22	TCMDC-	-0.25	110	TCMDC-	103.86	180

143370			143527			143261		
TCMDC-143360	88.36	25	TCMDC-143334	96.48	145			
TCMDC-143635	63.03	27						
TCMDC-142716	105.09	32						
TCMDC-143579	2.74	35						
TCMDC-143493	103.76	39						
TCMDC-143516	102.84	47						
TCMDC-143173	65.95	48						
TCMDC-143189	65.72	51						
TCMDC-143226	54.57	55						
TCMDC-143125	76.96	62						
TCMDC-143648	59.02	66						
TCMDC-143436	102.64	68						

TCMDC-143556	97.28	69						
TCMDC-143393	32.46	73						
TCMDC-143645	97.25	79						
TCMDC-143192	70.19	95						
TCMDC-143505	92.79	96						
TCMDC-143453	103.56	97						
TCMDC-143640	61.63	98						
TCMDC-143394	136.51	99						
TCMDC-143073	118.28	102						
TCMDC-143146	134.10	103						
TCMDC-143210	105.52	104						
TCMDC-143199	72.54	106						
TCMDC-	96.59	107						

143225								
TCMDC-143401	51.73	108						
TCMDC-143326	1.58	109						
TCMDC-143357	67.41	110						
TCMDC-143471	92.18	113						
TCMDC-143343	26.38	114						
TCMDC-143251	6.18	116						
TCMDC-143307	29.17	117						
TCMDC-142497	91.83	119						
TCMDC-143400	7.00	122						
TCMDC-143373	67.59	123						
TCMDC-143341	3.28	126						
TCMDC-143250	97.56	128						

TCMDC-143363	7.03	131						
TCMDC-143154	0.40	132						
TCMDC-143638	3.07	133						
TCMDC-143335	100.17	137						
TCMDC-143219	86.87	138						
TCMDC-143337	46.83	139						
TCMDC-143206	94.54	141						
TCMDC-143074	85.26	147						
TCMDC-143386	64.58	148						
TCMDC-143254	58.80	149						
TCMDC-143242	91.45	152						
TCMDC-143359	56.06	154						
TCMDC-	46.02	155						

143377								
TCMDC-143233	94.40	156						
TCMDC-143460	99.19	159						
TCMDC-143580	54.76	161						
TCMDC-143390	85.08	162						
TCMDC-143339	80.73	163						
TCMDC-143263	91.61	166						
TCMDC-143112	98.54	167						
TCMDC-143365	54.99	168						
TCMDC-143292	44.21	170						
TCMDC-143392	69.24	175						
TCMDC-143454	79.98	176						
TCMDC-143176	72.76	178						

TCMDC-143380	67.71	179						
TCMDC-143356	93.17	180						
TCMDC-143172	83.79	181						
TCMDC-143412	86.33	182						
TCMDC-143289	5.69	183						
TCMDC-143378	5.17	184						
TCMDC-143290	92.68	185						

Appendix 3 – AAT Target Product Profile

The AAT target product profile found at GalvMed. The TPP details the ideal and minimum attributes expected of a therapeutic treatment for AAT.

Attribute	Ideal TPP (Wants)	Minimum TPP (Needs)
1 **Active Ingredient**	Novel agent with new mechanism of action. No cross- or side-resistance to existing product actives.	Novel agent. Side-resistance to existing veterinary products acceptable if overcome by greater intrinsic potency or speed of action.
2 **Indication for use**	Treatment of *T. congolense, T. vivax, T. brucei* and *T. evansi* infections, including strains resistant to existing trypanocides.	Treatment of *T. congolense* and *T. vivax* infections, including strains resistant to existing trypanocides.
3 **Target species**	Cattle, sheep, goat & other ruminants, camels, horses, donkeys, pigs.	Cattle
4 **Route of administration**	Injectable (i.m. and s.c.) plus oral option for sheep.	Injectable (preferably i.m. or s.c.) or Pour-on or Oral.
5 **Formulation**	Injectable: Pre-formulated solution. Oral: Solid bolus or suspension/solution drench.	Injectable: Pre-formulated solution or suspension or sterile powder in vial for reconstitution. If injectable not possible then consider either pre-formulated pour-on or oral (solid bolus or liquid).
6 **Regimen**	Single administration.	Two administrations (provided the interval between the two administrations is short).
7 **Recommended time of treatment**	At first diagnosis of disease (clinical signs or parasitaemia).	At first diagnosis of disease (clinical signs or parasitaemia).
8 **Expected efficacy**	Absence of parasitaemia and improvement of clinical signs, e.g. anaemia. [For Regulatory Studies: Absence of clinically significant parasitaemia, e.g. buffy coat method for duration of regimen period, and normal haematocrit/PCV].	Absence of parasitaemia and improvement of clinical signs, e.g. anaemia. [For Regulatory Studies: Absence of clinically significant parasitaemia, e.g. buffy coat method for duration of regimen period, and normal haematocrit/PCV].

Attribute	Ideal TPP (Wants)	Minimum TPP (Needs)
9 **Target animal safety**	No clinically significant adverse drug reactions. Minimal administration site reactions. Safe for use in breeding animals (male and female) – demonstrated in target species.	No serious adverse drug reactions. Acceptable reactions at administration site. Safe for use in breeding animals (male and female) – demonstrated in laboratory animal studies.
10 **Withdrawal period**	Milk zero. Meat <14 days.	Milk <7 days. Meat ≤28 days.
11 **Special requirements for animals**	Compatible for concomitant use with common treatments e.g. ectoparasiticides, antimicrobials, anthelmintics & vaccines.	None stated.
12 **Special requirements for persons**	No special precautions required beyond good practice.	Routine personal protective equipment. No major hazard on accidental self-injection or pour-on.
13 **Special requirements for environmental protection**	No special precautions.	Minimal soil/water residues. Minimal restrictions on disposal of packaging.
14 **Package size**	1, 5, 10 and 50 doses packages.	1 and 10* doses. **Product price dependent*
15 **Price to user**	<US$2/dose (300kg animal).	Higher prices than US$2/dose are a major challenge unless justified by value-added properties and include syringes, etc.
16 **Storage requirements**	Ambient temperature ≤40°C/75% RH.	Ambient temperature ≤30°C/75% RH * **FAO/WHO Guideline*
17 **Shelf-life as packaged**	≥3 years*	≥ 18 months* ** At world zone IVb for regulatory purposes*
18 **Shelf-life after first opening**	≥7 days & <28 days	≥24 hours for single-dose ≥7 days for multidose vial