Diamidine Transport in the Kinetoplastidae.

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July 2003

Summary

African trypanosomes are protozoan parasites that are unable to synthesise purines *de novo* and as a result must scavenge them from the host environment. Purines are transported into the cell via a number of surface membrane transporters that recognise specific motifs on the substrate. These transporters can be exploited to mediate the uptake of trypanocidal drugs, such as diamidines and arsenicals. The high affinity pentamidine transporter 1 (HAPT1) and the low affinity pentamidine transporter1 (LAPT1) are involved in the uptake of the diamidine compound pentamidine and the specificities and associated affinities of these transporters for various other compounds were tested by looking at the inhibitory effect of varying concentrations of test drugs on the uptake of ^{3}H-pentamidine.

A link was found between the carbon chain length of diamidine compounds and their affinity for HAPT1 and LAPT1. For compounds containing benzene and furan rings, the position of methyl groups in relation to these structures also appears to be important. HAPT1 and LAPT1 differ in ligand recognition profiles, although both are implicated here in the uptake of Isometamidium and Ethidium Bromide, two trypanocidal drugs previously thought to be taken up by diffusion only. As there is increasing drug resistance to all trypanocidal drugs, HAPT1 and LAPT1 are extremely important as alternative delivery systems for trypanocides in the event that other transporters are lost in the field through drug pressure, for example P2.

The P2 transporter known to mediate the uptake of adenosine and melaminophenylarsenicals is shown here, by using transport assays in conjunction with Michaelis-Menten kinetics, to be the sole mechanism for the uptake of veterinary drug Berenil, in *Trypanosoma brucei brucei*. Finally, Crithidia fasciculata, another kinetoplastid, is shown to have particular attributes, which would value this organism as a potential expression system for the characterisation of HAPT1 and LAPT1.

Acknowledgments

I Laura Fay Anderson declare that this is all my own work .

Contents Page

Tables and Figures

Title	**Page Number**

Chapter 1

General Introduction

African Trypanosomes and trypanosomiasis

Classification, morphology and the life cycle

African trypanosomes are part of the order Kinetoplastida, which encompasses a group of parasitic protozoan organisms, all possessing a unique organelle, the kinetoplast. This structure is found within the mitochondrion near the flagella and contains its own DNA which consists of a network of about 20 000 mincircles and 20-50 maxicircles. (Roberts and Janovy, 1996). Trypanosomes belong to the Trypanosomatidae family and members of this group, for example *Leishmania* and *Crithidia*, share morphological, biochemical and metabolic attributes. Trypanosomatids are usually pathogens of humans and animals where they reside in the blood of the vertebrate, definitive host and are transmitted by bloodsucking invertebrates.

The two species of trypanosome that infect humans, *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* are morphologically indistinguishable. They are heteroxenous and are transmitted by the tsetse fly of the genus *Glossina*. (Figure 1A: Life cycle). The trypanosome is pleiomorphic in the human host. The long, slender, flagellated dividing form, which consumes large quantities of glucose and oxygen, differentiates to the short stumpy infective form, that lacks a free flagellum and has a pronounced undulating membrane. The latter form is ingested by the tsetse fly in a blood meal and multiplies in the posterior of the midgut for about 10 days before migrating to the salivary glands and transforming into epimastigotes. These are metacyclic trypomastigotes which will divide several times, usually by binary fission (although genetic recombination of phenotypes has been demonstrated) before being transmitted
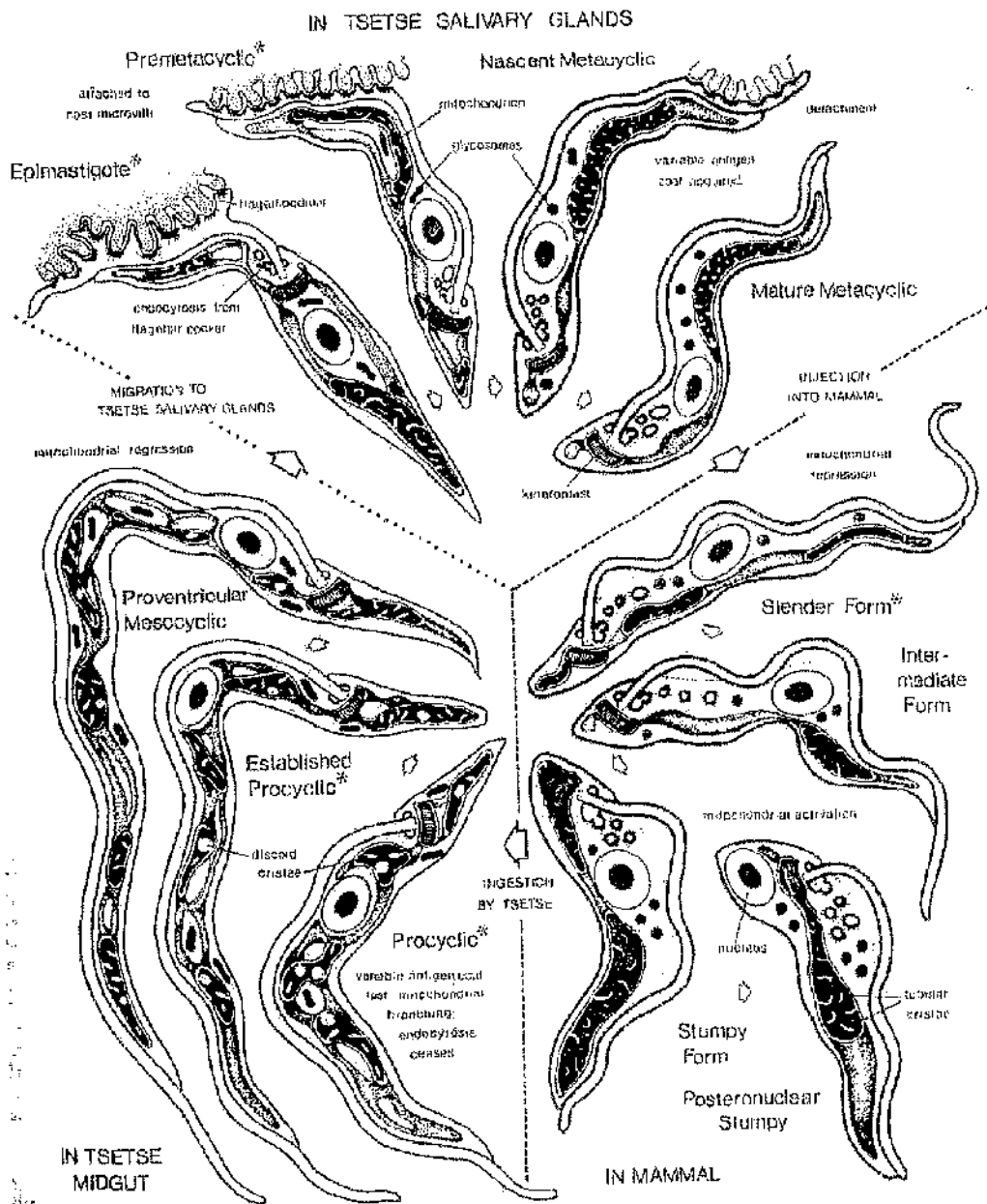
Figure 1A. The trypanosome life cycle.
Reproduced from Vickerman, 1985.

3

back to the definitive host by the vector, which acts as a syringe, injecting up to several thousand parasites in a single bite. It is thought that these haemoflagellates originally parasitised the digestive tracts of insects and were able to establish themselves in the vertebrate host by adapting morphologically and physiologically to cope with the new environmental conditions of blood (Hoare, 1967).

Pathology and Disease

Trypanosomiasis or sleeping sickness presents itself differently in patients depending on whether *T.b.gambiense* or *T.b.rhodesiense* is the source of infection. The former causes a chronic form of the disease with the patient progressively degenerating over a period of about two years from the point of transmission to death. However, in the case of *T.b.rhodesiense* infection, symptoms are more severe and death may occur within weeks of contracting the pathogen. Generally, there are two stages to the disease. The acute phase begins as a flu-like illness, with mild symptoms such as headache, joint pains and an irregular fever. At this stage parasites can be detected in the bloodstream of the infected person. They are therefore fully exposed to the immune cells present in the blood and have evolved a strategy to evade the humoral immune response. The trypanosomes replace variant surface glycoproteins, which form a layer on the surface of the protozoa, with new proteins expressing a different antigenic profile. This is known as antigenic variation and leads to fluctuating parasitaemias, as the body takes about one week to mount an immune response to each new antigen. Therefore, symptoms also fluctuate in correlation with this phenomenon. As the disease progresses, symptoms

4

become more severe and patients may experience behavioural problems, mental retardation, lethargy, anaemia and emaciation, which is more pronounced in *T.b.rhodesiense* sleeping sickness (Figure 1B).

In late stage sleeping sickness the parasites infect the central nervous system (CNS) and can be found in the cerebrospinal fluid (CSF). Progressive destruction of neurones occurs in cortical areas of the brain and patients may experience a plethora of associated neurological disorders such as tremors, involuntary movements, low and tremulous speech and a variety of psychological disorders. Gradually, the patient becomes more difficult to rouse from sleep and develops meningo-encephalitis leading to coma and ultimately death, if left untreated (Neva and Brown, 1994).

As the early stage symptoms of trypanosomiasis are similar to those of many other common diseases found in Africa, it is difficult to diagnose a patient from these alone. Trypanosomes can be detected in the blood using geimsa stain or ELISA and by examining CSF using the lumbar puncture method in late stage of the illness.

Epidemiology

There are now an estimated 400 000 people harbouring trypanosomiasis throughout 36 countries in sub-Saharan Africa. (Barrett, 1999). The area at risk of disease is dependant upon the distribution of the tsetse fly vector (Figure 1C) and is commonly known as the tsetse fly belt. *T.b.gambiense* (West African) and *T.b.rhodesiense* (East African) are transmitted by different species of tsetse fly, which reside in contrasting habitats (riverine and savannah, respectively) causing these species to be prevalent at opposite sides of the continent with the Rift Valley in the centre. *T.b.gambiense* is largely endemic in West

Figure 1B: Sleeping sickness patient

Figure 1C: Tsetse fly distribution indicated in red
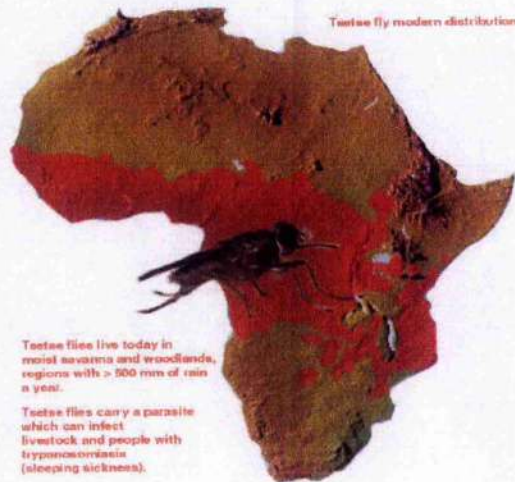
Figure 1D: *Crithidia fasciculata*
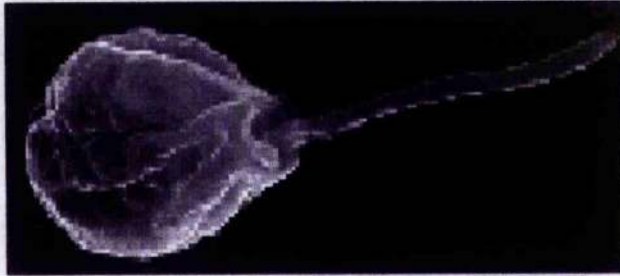
Africa and can be contracted at work or home whilst collecting water from wells or rivers. *T.b.rhodesiense*, on the other hand, is a zoonotic, sporadic disease affecting particular occupational groups who spend time with wild or domestic animals. However, *T.b.gambiense* has also been discovered to be an important zoonotic pathogen in West Africa, for example Nigeria, in areas where livestock is reared (Coker et al, 2000). In 1960 Human African Trypanosomiasis (HAT) had disappeared almost completely but since then the number of cases have been steadily rising and there are now epidemics in Southern Sudan, Uganda (Welburn et al, 2001) and the Democratic Republic of Congo. There are several reasons for the massive increase in cases of HAT. The colonials left Africa around the late 1950's without leaving behind health policies, education and other control methods, previously used by them to combat the disease. Gradually, the fly populations returned to the areas where they had been eradicated. Politics also plays an important role, where politically unstable countries are unable to persist with national and local public health control programmes (Goodwin, 1964). In the event of war, which has been common in the afflicted areas, money is spent on other resources and the large scale migration of people with parasites exposes a new population to the disease.

Bovine Trypanosomiasis

The three most important species of trypanosome that infect cattle are: *Trypanosoma congolense, T.vivax* and *T.b.brucei*. The species most commonly acquired in East Africa is *T.congolense* and *T.vivax* in West Africa. *T.b.brucei* is not limited to a particular area and was the first species of trypanosome to be discovered in the blood of infected cattle by Sir David Bruce. All species cause the devastating disease nagana, named by the Zulu people, meaning "poorly". Symptoms include anaemia, indicated by a reduction in packed cell volume (Bossche and Rowlands, 2001), oedema, watery eyes, fever and spontaneous abortion (Geerts and Holmes, 1998). Within months cattle become emaciated, uncoordinated and paralysed which eventually leads to death. Parasites can be detected in the blood and characterised using PCR, which is more reliable than ELISA and microscopic methods (de Almeida et al, 2001, Rebeski et al, 1999, Mugittu et al, 2001, Desquesnes et al, 2001). Vascular damage is found in chronic *T.congolense* infection, probably because these trypanosomes attach to small blood vessels whereas *T.b.brucei* does not. However, *T.b.brucei* leaves the blood vessels and enters tissues to produce an inflammatory reaction whereas *T.congolense* is strictly a plasma parasite (Losos et al, 1973).

The severity and duration of disease is dependant upon the susceptibility of the host. Certain taurine breeds of cattle indigenous to West Africa, the N'Dama, Maturu and Dahomey, are resistant or partially resistant to trypanosomiasis (Paling et al, 1991). Although these cattle will still present symptoms, death is rare and they can control a rechallenge infection more efficiently than trypanosusceptible Boran breeds. Trypanotolerance is thought to be under genetic control and this may affect a hosts'

8

ability to control disease through immune mechanisms (Taylor, 1997). It would therefore be advisable to breed trypanotolerant cattle which will thrive in areas of tsetse infestation. However, these breeds, although immunologically stronger do not necessarily have the other desirable qualities required to make a sufficient profit on the market.

The tsetse fly area covers about 4 million square miles and it is thought that if the land could be used to raise cattle, the present cattle population in Africa would increase to 125 million, almost double the current amount. At present there are about 45 million cattle forced to be raised under challenge from trypanosomiasis and 6 million of these alone are found in the Ghibe Valley, Ethiopia (Itty et al, 1995). In Tanzania between 1982-86 74 818 cattle died from trypanosomiasis and at present 70% of the country is devoid of cattle (Mbwambo et al, 1988). It is estimated that US $12 000 000, related to cattle production in sub-Saharan Africa, is lost every year and this does not take into account losses made through manual labour, which is 80% in most areas, due to the lack of draught power (Taylor, 1997). Animal power has been extremely important in the rural and economic development of Asia and Europe, something Africa has dispossessed. The inability to rear cattle and practise livestock farming due to tsetse infestation prevents economic development of these areas through depriving the people of a productive meat and milk trade and leading to poverty. Huge losses will also be made every year on drugs to treat trypanosomiasis. As a solution farmers graze their cattle at higher altitudes where tsetse flies are scarce. However, this is often far from abattoirs and meat markets, forcing the farmers to drive cattle for weeks through tsetse-infested land without medicine, leading to unaccountable deaths in the herd (Jordan, 1986). However, it has recently been suggested that if farmers in areas of high trypanosome risk pay higher prices for drugs

and full veterinary costs they should still be able to make good profits on their stock (Itty et al, 1995). This does not eliminate that fact that by being forced to pay these prices, these people are still economically disadvantaged.

Control of Trypanosomiasis

The current focus on control of trypanosomiasis in humans and animals is by chemotherapy. There is little hope of a vaccine due to lack of vaccine candidates. Antigenic variation makes it virtually impossible to vaccinate a host against a surface antigen and cattle recently vaccinated against parasite specific flagellar pocket antigens were only partially protected against rechallenge with *T.congolense* and *T.vivax* (Taylor, 1997). The other solution is to eradicate fly populations through methods of vector control. Mono- or Biconial fly traps baited with insecticides have been successful in reducing population numbers but people see little long term benefits and tend to dismantle the traps for use in social activities such as fishing. There are several insecticides available. DDT has been used for ground spraying on a large scale but this is not viable during wartime conditions and it has also been found to have detrimental effects on the environment and surrounding wildlife. Pyrethroids have been used as cattle dips, which are less toxic and generally more flexible. However, there is an increasing problem of insecticide resistance (Allsopp, 2001, Grant, 2001). Other methods include clearing of breeding sites and elimination of animal reservoirs, both of which are

destructive and time consuming and the movement of people out of infected areas is unpopular. A simple solution would be to graze cattle at night to avoid contact with the vector host. A novel idea is to release flies infected with an insect pathogen affecting the germ line, which will ultimately eliminate the residing population (Aksoy, 2001). However, a lot of research and ethical discussion must be carried out before this method becomes plausible.

Therefore, the only option left is chemotherapy. Although there are a number of drugs currently on the market for treatment of HAT and bovine trypanosomiasis these have several problems associated with them.

Chemotherapy

Drugs for treatment of HAT

There are four drugs available for treatment in humans: Suramin and Pentamidine used for treatment of early stage sleeping sickness caused by *T.b.rhodesiense* and *T.b.gambiense*, respectively. For late stage sleeping sickness Melarsoprol is the first drug of choice followed by DFMO (Difluoromethylornithine) if necessary. The modes of action of these drugs and the problems encountered are very different and these will now be discussed individually in some detail.

11

Suramin (Moranyl®)
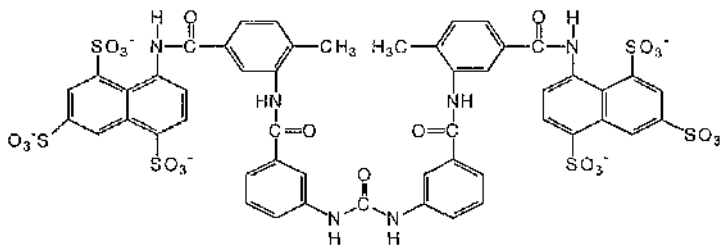
Suramin, a polysulphonated symmetrical naphthalene derivative, was developed by German chemists and introduced for treatment of early stage sleeping sickness in 1922. At the time a number of naphthalene dye substances had been shown to have trypanocidal activity and therefore this compound was synthesised with the intention of being a trypanocide. Although the drug has now been in use for 80 years the mode of action is still unknown. The drug cannot be used to treat late stage sleeping sickness, because with six negative charges at physiological pH, it cannot cross the blood brain barrier and therefore only removes trypanosomes from the bloodstream. Toxic side effects, such as vomiting, shock and fatal hypersensitivity reactions occur in about 1 in 20 000 cases. However, Suramin is also the main drug used against the filarial parasite *Onchocerca volvulus*, which is responsible for river blindness. Recently, the pharmaceutical company Bayer planned to stop production of the product, probably due to the lack of an attractive profit making market, and it would no longer be available if the WHO had not interceded (Barrett, 1999).

Pentamidine (Lomidine®)

$$H_2N \diagdown C(-H_2N) \diagup \bigcirc \diagup O-(CH_2)_5-O \diagup \bigcirc \diagup C \diagdown NH_2 (NH_2)$$
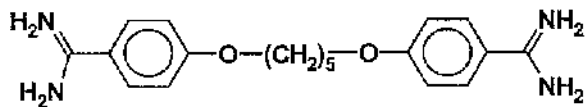
Pentamidine (1,5-bis (4'-amidinophenoxy) pentane) is an aromatic diamidine, synthesised in 1937. It is only effective at clearing trypanosomes from the bloodstream and therefore is no use in late stage sleeping sickness. However, it has recently been found that pentamidine can, surprisingly, cross the blood brain barrier (BBB) and is detected in the cerebrospinal fluid (CSF) (Bronner et al, 1991). Perhaps this is due to the breakdown of the BBB by the inflammatory response caused by trypanosome invasion of the CNS. Pentamidine is not well absorbed orally and therefore must be given intravenously or by subcutaneous injection. When administered like this the drug is stored within the blood and tissues to create a prophylactic effect, protecting the individual for up to six months, and has been used on a mass scale in past epidemics (Goodwin and Rollo, 1955). A suckling child can also gain enough pentamidine from mothers' milk to protect he/she from the disease (Williamson, 1962). However, the drug and other related diamidine compounds have a vasodepressor effect (Wien, 1943) and as a result can cause toxic side effects. Adverse reactions occur in about 50% of patients. Symptoms include swelling and abscesses at the sites of injection, hypoglycaemia progressing to diabetes mellitus and hyperkalemia (an excess of potassium in the blood) suggesting an impairment in renal function (Kleyman et al, 1995). It is found that combining Pentamidine and Suramin helps to alleviate these painful symptoms (Gumaraes and Lourie, 1951).

13

Pentamidine accumulates to 1 mM in the trypanosome before lysis occurs which is a surprisingly high concentration. The compound has various drug targets such as S-adenosyl-L-methionine carboxylase, (although not involved in trypanocidal action)(Berger et al, 1993) mitochondrial topoisomerase II, Ca^{2+} transport (Benain et al, 1993) and lysine-arginine transport (Berger et al, 1995). It has also been found to restrict the replication of a murine rotavirus by acting as a protease inhibitor (Vonderfecht et al, 1988). The main mode of action is thought to be by binding to the minor grooves of DNA in the kinetoplast causing 5% cleavage in the minicircles (Wang, 1995). Trypanosomes exposed to pentamidine become dyskinetoplastic but still retain their mitochondrial membranes. Most drugs with affinity for DNA are also mutagenic. However, pentamidine is Ames negative (Stauffert et al, 1990), and there is absolutely no effect on nuclear DNA.

In the 1940's pentamidine and the related diamidine compound propamidine, were shown to have potent bacteriocidal activity. As a result, propamidine can be used in topical application to prevent streptococci and staphylococci infection of wounds and burns, with no toxic side effects (Fulton, 1943). It also shows some fungistatic action (Elson, 1945).

Pentamidine has also shown activity against a variety of other organisms such as *Plasmodium falciparum* (the malaria causing parasite) (Bell et al, 1990, Stead et al, 2001). *Candida albicans, Babesia canis* and can be used as an alternative to allopurinol for treatment of Leishmaniasis (Croft and Brazil, 1982). Most importantly it shows activity against *Pneumocystis carinii* that causes pneumonia by affecting oxidative phosphorylation and nucleic acid synthesis (Goa and Campoli-Richards, 1987). This opportunistic pathogen was rare before 1981 but due to the emergence of AIDS the

disease is now particularly prevalent in the US. Pentamidine was licensed for the treatment of *Pneumocystis carinii* in 1985 (Pearson and Hewlett, 1985). The manufacturers, Rhône-Poulenc, then raised the price of the life saving sleeping sickness drug, for use in the Western World and a special deal had to be negotiated by WHO to allow the drug to remain available to Africa (Barrett, 1999).

Melarsoprol (Arsobal®)
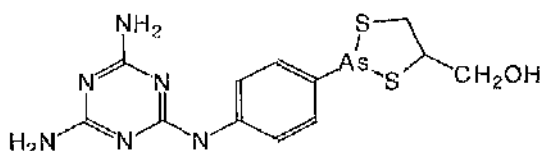
Melarsoprol is a trivalent melaminophenyl arsenical introduced in 1949 by Friedheim for the treatment of early and late stage sleeping sickness. It is active against both *T.b.gambiense* and *T.b.rhodesiense* infection. The drug is lipid soluble allowing it to cross the BBB and is therefore the current drug of choice for the treatment of late stage sleeping sickness. However, it has recently been discovered that surprisingly low levels of melarsoprol are detected in the CNS (Pepin and Milford, 1994). Perhaps this is because the drug is rapidly converted to the less permeable melarsen oxide and other compounds in the body (Barrett, 2000). The solid compound is unstable and the drug is therefore marketed as a solution. However, this still deteriorates soon after opening. Also, the solvent propylene glycol, used to form the solution, is an irritant and causes extreme pain when administered by intravenous injection (Jennings, 1993). The drug causes rapid

lysis of trypanosomes but the mode of action is unknown. Melarsoprol has also shown activity against leukaemia and myeloma cells in vitro (Pepin and Milford, 1994).

There are several severe problems associated with this drug. Granulomatous tissue often forms at the site of injection but more importantly, about 5% of patients treated with melarsoprol suffer from a fatal post-treatment reactive encephalopathy (PTRE). However, without treatment there is 100% mortality and so there is no choice in accepting this painful and potentially life threatening chemotherapy (Kaminsky and Mäser, 2000). There are various suggestions as to why these encephalopathies occur in some patients. It is possibly due to an excess of arsenic in the body. However, it appears that anti-inflammatory drugs, such as Prednisolone, helps to reduce the incidence of PTRE which suggests that the problem may come from an inflammatory response directed at trypanosomes which remain in the CNS (Jennings, 1993). Or it may be possible that the release and binding of trypanosome antigens to the brain cells leads to recruitment of immune cells into the CNS. This will allow the formation of immune complexes and will trigger a massive inflammatory reaction directed towards the brain tissue (Pepin and Milford, 1991). In either case, it is important to eliminate all trypanosomes from the CNS as quickly as possible or to catch the illness in early bloodstream stage before the CNS has become infected.

Another increasing problem associated with the drug is relapse of infection after treatment with melarsporol, a rare occurrence after treatment with Suramin or Pentamidine. This occurs in up to 17% of people treated and these patients will die unless given an alternative drug. This phenomenon has been reported since 1960 and is common in areas of epidemics. In Northwest Uganda, 1% of the population are infected with

T.b.gambiense and there have been huge increases in the rates of relapses (now almost 30%) in this area. A study showed that this might be due to the parasites having reduced sensitivity to the drug (Matovu et al, 2002). Other factors may play a part in relapse, such as the patients' metabolism (causing faster degradation of the drug) and the affinity of parasites for extravascular sites in the CNS that cannot be accessed by the drug (Brun et al, 2001).

Eflornithine (DL-α-difluoromethylornithine) / Ornidyl®

$$F_2HC-\overset{\displaystyle COOH}{\underset{\displaystyle \underset{\displaystyle \underset{\displaystyle \underset{\displaystyle NH_2}{\overset{|}{CH_2}}}{\overset{|}{CH_2}}}{\overset{|}{CH_2}}}{\overset{|}{C}}}-NH_2$$
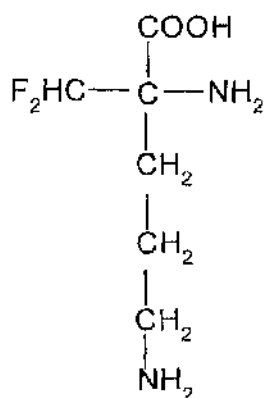
Eflornithine, (DFMO) was introduced in 1990 for the treatment of late stage sleeping sickness and is also known as the resurrection drug for its ability to bring people back from the brink of death. High levels can be found in the CSF demonstrating the drug's ability to penetrate the BBB. It is usually used as a secondary treatment when melarsoprol fails but is only effective against *T.b.gambiense* infection, as *T.b.rhodesiense* appears to have an innate tolerance to the drug. The compound works by blocking the polyamine pathway at ornithine decarboxylase. The polyamine pathway has a central role

in cell proliferation and therefore this is not only a drug target in trypanosomes but also in cancer cells, which proliferate indefinitely. Eflornithine was therefore originally synthesised as an anti-cancer reagent but was found to be particularly active against trypanosomes (Barrett and Barrett, 2000).

Associated problems include drug administration, the extremely large doses of drug required and the cost at an estimated $750 per person.

Combination Chemotherapy

Essentially, all treatment given at late stage sleeping sickness is regarded as combination chemotherapy because Suramin or Pentamidine are still used to clear bloodstream infection in conjunction with Melarsoprol, to treat the CNS infection. However DFMO and Suramin only produce a 70-80% cure rate and DFMO and pentamidine together are not successful. Pentamidine also targets the polyamine pathway, like DFMO, but has the different target of S-adenosylmethionine decarboxylase. The binding of pentamidine to this enzyme is reversible, which may provide a reason for an unsuccessful cure when combined with DFMO (Jennings, 1992). The intake of DFMO was increased by 50% if mice were kept in the light for 2 hours and then transferred to the dark for 4 hours. Clinical trails following this method are now being carried out on humans.

Nufurtimox/ Lampit is a drug used for the treatment of Chagas disease caused by the South American trypanosome, *T.cruzi*. It has also been given in combination with melarsoprol or DFMO to treat African trypanosomiasis (WHO, 1998). However, patients respond poorly to this treatment due to bad side effects such as skin rashes, headache,

gastro-intestinal problems and weight loss (Nieuwenhove, 1992). Supervision is required to ensure the course of treatment is completed (Jennings, 1993).

Development of New Drugs

To develop new drugs it is important to identify parasite specific drug targets and as some of these for trypanosomes are known, for example the polyamine pathway, drugs can be synthesised to target particular essential biochemical pathways in the parasite without disrupting the metabolism of host cells. Another important aspect of drug design is to improve the uptake of drugs into the target cell, which has received little attention in the past ten years (Croft, 1999).

The major problem concerning the development of trypanocidal drugs is finding a way to get drugs to penetrate the BBB to treat late stage infection. The BBB is made up of endothelial cells joined by tight junctions. Cerebral capillaries prevent the entry of polar solutes into the brain. The components of the CSF are regulated by transport systems that allow the entry of amino acids, glucose and macromolecules whilst lipophilic compounds are allowed to passively diffuse across membranes. This presents a problem when trying to deliver drugs to this area of the body. High sugar solutions have been found to briefly open the intracellular junctions, which allow antitumour drugs to cross the barrier (Neiwelt and Barnett, 1989). However, recently the development of drugs for other diseases of the CNS found in the West, for example Alzheimers' and Parkinsons', have focused attention on research to improve the delivery of drugs across the BBB (Croft, 1999).

At present there are no new compounds for trypanosomiasis in phases 2-3 of clinical trials. Drugs must pass efficacy, toxicity and mutagenicity testing before being tested on animals and eventually humans and most are withdrawn at an early stage. Due to the introduction of new safety regulations new drugs now take even longer to be approved. The trypanocidal activity of DFMO was first published in the early 1980s' and yet wasn't legally registered in Europe until 1991 (Keiser et al, 2001). Also pharmaceutical companies have lost interest in the development of new drugs, as they will only be useful in developing countries, which don't constitute an attractive profit making market, unless the drugs are effective against a wide range of organisms that can also be found in the western world. A Pan-African licensing agency may allow African pharmaceutical companies to see the benefits of developing drugs of local importance which will in turn show western companies that these drugs can be developed for a lot less than the US$300 million the estimated annual cost (Barrett, 1999). Many of the drugs currently in use, with the exception of DFMO, were introduced onto the market before licensing laws were tightened up in the 1970s' and therefore the majority probably wouldn't pass the safety tests of today. However, the consequences of no treatment outweigh those associated with drug risks. Perhaps an alternative to new drugs would be to improve the drugs already available.

Veterinary Drugs

There are three drugs available on the market for the treatment of bovine trypanosomiasis. These are commonly known as Homidium, Berenil and Samorin. These

are effective against *T.brucei brucei*, *T.congolense* and *T.vivax* infection in cattle, sheep and goats. They are popular because not only are they effective but also easily affordable. It has also been suggested that chemotherapy reduces the pathogenicity of particular strains (Losos and Ikede, 1972). I will now discuss the modes of action and problems associated with these drugs independently.

Diminazene aceturate/Berenil®
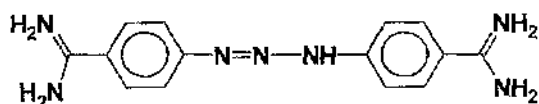
Diminazene, the active component of Berenil®, was synthesised by Jensch in 1955 as a trypanocide for domestic livestock and is the most widely used therapeutic agent for the treatment of bovine trypanosomiasis. This compound is an aromatic diamidine, derived from Surfen C and is an analogue of pentamidine. The two compounds have very similar modes of action. Berenil also binds irreversibly to the minor groove of DNA probably via electrostatic and hydrogen bonds due to its charged terminal amino groups and forms interstrand crosslinks (Gonzalez et al, 1999). It appears to have a higher affinity for AT rich portions of DNA and therefore binds more readily to 5'-AATT-3' regions than 5'-TTAA-3' (Den Bossch, 1972). However, it does also bind to GC rich regions of DNA but the bonds are weaker (Barcelo et al, 2001). Berenil also has DNA intercalating properties (Reddy et al, 1999) and acts as a topoisomerase II inhibitor in trypanosomes to cause

21

cleavage in the kinetoplast minicircles, at a concentration of 1μM. This is perhaps why it can produce dyskinetoplastids. In *T.cruzi* the binding of Berenil to AT rich initiation sites blocks replication of circular kinetoplastid DNA (Brack and Delain, 1975). A study showed that *T.brucei brucei* are irreversibly damaged after being exposed to diminazene for 1 minute at concentrations equal to those in the plasma of cattle receiving chemotherapy (Kaminsky et al, 1993). A strange phenomenon is that Berenil appears to release *T.congolense* into the blood stream from the microcirculation, where this species is usually found attached to endothelial cells (Maxie and Losos, 1977). It is suggested that Berenil does not kill the trypanosomes but makes them susceptible to the hosts' defences. However, this has now been shown to occur with other trypanocides and it appears that the higher the number of parasites released into the jugular vein, the quicker the parasites are cleared from the bloodstream (Kalu and Aina, 1984).

The diamidine compound is marketed as a solution with the stabiliser antipyrine to prolong activity for 10-15 days in storage. The molecular structure consists of a triazene bridge linking two amidinophenyl moieties (Peregrine and Mamman, 1993). The drug is administered by intramuscular or intravenous injection at a recommended dose of 3.5-7.0 mg^{-1} kg per body weight. However, higher doses are often required to treat *T.brucei brucei* infection (Peregrine, 1994). High doses are relatively well tolerated, as there are no toxic side effects associated with the compound in cattle (Peregrine and Mamman, 1993, Fairclough, 1963). On administration the drug is rapidly dispersed from the site of injection to the plasma and lymph (Mamman et al, 1996) and then accumulates in the liver and kidneys (Kellner et al, 1985, Mdachi et al, 1995) but the majority is rapidly excreted in the urine within 24 hours and is therefore useless as a prophylactic. The drug

can be detected in the milk of sheep and goats ten minutes after administration (El-Banna et al, 1999). and is also found at a high level in cows milk 8 hours after administration. This is a concern because the drug may be bioavailable to the consumer (Mdachi et al, 1995). Also, a calf suckling from a mother treated with Berenil could be exposed to sub lethal doses of the drug, which may contribute to the development of drug resistance. It may also be possible that the trypanocide residues are also present in meat products and could affect the consumer. It is therefore suggested that a drug withdrawal period of 28-35 days is recommended, before slaughter takes place, to minimise the consumers exposure (Aliu et al, 1984). In areas of high trypanosome challenge it would be too expensive to use Berenil as a sole treatment and therefore it is often used alongside other trypanocides. Berenil has also been found to prevent the establishment of infection in the tsetse flies after a blood meal has been taken from cattle treated with the drug (Diack et al, 1997). In flies that do obtain infection, trypanosomes can be found in their midgut but reduced numbers develop a salivary infection and therefore cannot transmit the parasite to another host. The same is true for treatment with Samorin (Jefferies and Jenni, 1987).

One of the major problems with Berenil is that it does not easily cross the BBB and therefore cannot clear trypanosomes from the CNS. This may account for relapse of infection, common in animal trypanosomiasis, as therapeutic drugs cannot target trypanosomes that remain in the CNS. It has been found that lithium chloride, which is a hyperosmolar agent, can cause openings in the endothelial cell junctions. This allows Berenil to penetrate the BBB at therapeutic concentrations (Odika et al, 1995). PCR can now be used as a sensitive method for monitoring the efficacy of diminazene and for

the detection of remaining parasites in the CNS (Bengaly et al, 2001, Clausen et al, 1999).

Diminazene can also be used as a babesicide in cattle and sheep, which adds an advantage of veterinary usage. In fact, its original name was Babesin. It shows good activity against *Babesia canis* in particular and has been used to successfully control this infection in dogs. However, care must be taken not to administer high doses due to toxic side effects such as nausea, vomiting, nervous disorders and anaphylactic shock caused by the over-stimulation of the parasympathetic nervous system (Milner et al, 1997, Peregrine, 1994). Diminazene has also shown activity against cutaneous and visceral leishmaniasis (Pevers and Killick-Kendrick, 1987).

It is not recommended for use in humans due to toxic side effects experienced by a few animal species, namely dogs, camels and horses. However, although diamidines are DNA intercalating agents they appear to be non teratogenic. They are negative in the Ames test for mutagenicity but have been shown to have mutagenic effects on *Saccharomyces cerevisiae*. A long-term study has been carried out demonstrating the effects of Berenil on humans. The study began in 1965 and patients were treated for *T.b.gambiense* and *T.b.rhodesiense* infections with the drug. Immediately after administration patients experienced numbness in legs, paralysis, nausea, vomiting and pain in the soles of feet. However, none of these effects were permanent and all patients recovered fully. These side effects, with the exception of paralysis, are experienced when treated with Suramin, which is also a teratogen. Perhaps Berenil could be used as an alterative for the treatment of early stage sleeping sickness (Abaru et al, 1984). It cannot

be used if the CNS has become infected because this is associated with meningoencephalitis (Jennings, 1992).

Homidium (bromide or chloride)/Ethidium®
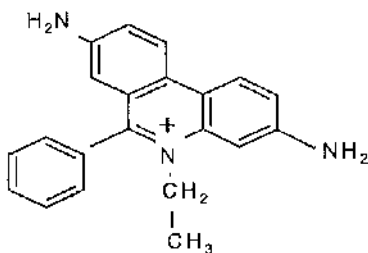
Homidium is a phenanthridinium compound that was introduced in 1952 by Watkins. It can be prepared as either bromide or chloride salts, as both have equal trypanocidal activity (Peregrine, 1994). However, ethidium bromide is only soluble in boiling water, which may be disadvantageous in the field whereas ethidium chloride can be dissolved in cold water (Williamson, 1962). The drug is administered via intramuscular injection because adverse reactions occur if given subcutaneously. It was originally used to stain nucleic acids because of its ability to intercalate between DNA and its fluorescent properties. Browning first demonstrated the trypanocidal activity of phenanthridinium compounds in 1938. It has been found to affect glycosomal function, trypanothione metabolism and replication of minicircles in the kinetoplast (Steinert, 1969) and as an intercalating agent it also blocks the replication of nucleic acids. However, homidium is a potent mutagen (Macgregor and Johnson, 1977, McCann et al, 1975), which may cause problems because under drug pressure a mutagen can induce mutations in the target cell

and select for genes that confer drug resistance. It was most widely used between the 1960s' and 1970s' (Wang, 1995) but has since been removed from the market in some areas due to an increase in drug resistance. The drug is eliminated rapidly from the body making it a poor prophylactic. It also causes depression of the respiratory centre in monkeys to cause loss of consciousness. (Hawking, 1963b) However, it also demonstrates antibacterial activity that is increased if cationic ionisation is induced. However, it appears that the structure must be planar to have powerful antibacterial properties (Albert et al, 1949).

Isometamidium chloride/ Samorin®
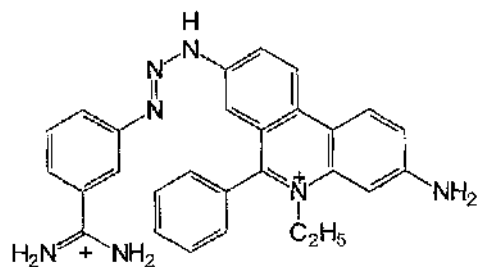
Like homidium, Isometamidium (ISMM) is a phenanthridinium compound and is identical in structure apart from the addition of an *m*-amidinophenyl-*azo*-amine moiety (Kinabo, 1993). This is in fact part of the Berenil molecule and therefore ISMM can be thought of as a conjugate of homidium and Berenil. Therapeutic trials were first carried out against *T.congolense* in mice (Wragg et al, 1958) and Berg introduced the drug onto the market in 1960. The drug is marketed as 70% ISMM and 30% isomers, one of which

26

includes homidium, and is found in the form of a dark red powder that has 6% solubility in water at 20°C. The trypanocidal mode of action is similar to both homidium and Berenil, by acting as an intercalating agent of DNA and blocking nucleic acid synthesis (Wilkes et al, 1995). The kinetoplast is thought to be the principal target as this is where most of the drug accumulates in the trypanosome. It also promotes cleavage of the minicircles in kinetoplast DNA (Shapiro and Englund, 1990). This would explain its specificity towards trypanosomes and not the host cell. ISMM has 10-20 times more activity towards *T.congolense* than *T.rhodesiense* (Hill, 1965). However, it appears to be acutely toxic to mammals, a feature which is not observed in the other two trypanocides. Abscesses often develop at the site of injection.

The recommended doses of 0.50-1.0 mg^{-1} kg are too toxic for routine use; however, Samorin is the only veterinary drug to exhibit prophylactic properties. A single dose can protect an animal for up to 7 months from infection (Wilkes et al, 1995). However, in areas of high tsetse fly challenge a single dose may only protect cattle for up to five weeks (Stevenson et al, 1995). Also, the efficacy of the drug treatment is found to be affected by the size of the initial inoculum of trypanosomes, or rather the degree of parasitaemia in the host blood (Sones and Holmes, 1992). After being administered by intramuscular injection the drug is rapidly dispersed throughout the body tissues, accumulating in the liver, kidney and spleen. However, a large amount of the drug binds to the injection site and is released slowly from this location to exert the prophylactic effect (Kinabo, 1993). The duration of protection is dependant on drug susceptibility of different trypanosome populations and the drug dose concentration administered to convey protection. A higher drug dose appears to protect cattle against subsequent

27

challenge for a longer period (Peregrine et al, 1988). The main route of elimination from the body is through excretion in the faeces. It is unclear how the drug is eliminated from the body, as excretions in the urine and milk are low (Murilla et al, 1996). It has also been found that Samorin prevents transmission of *T.congolense* to the tsetse fly vector and therefore acts as a transmission-blocking drug (Jeffries and Jenni, 1987).

Future developments

There are currently no new veterinary trypanocides being produced by pharmaceutical companies (Kinabo and Bogan, 1988). Instead recent attempts have been made to improve the drugs that are already available. Problems with toxicity have been reduced by complexing diminazene and isometamidium with dextran sulphate (De Dekin et al, 1989) have devised a cylindrical device that releases homidium at a slow rate when implanted in the body of rabbits. It appears to increase the prophylactic effects of the drug, protecting rabbits for up to 300 days, with no side effects involved. This would be useful in areas of constant tsetse challenge (Kinabo, 1993). However, if the body weight to device size ratio were to remain constant the contraption would be impractically large in cattle (Peregrine, 1994).

Finally, Quinapyramine (Figure 1E), a drug withdrawn from the market in 1976 due to widespread drug resistance has now been reintroduced onto the market for treatment of *T.evansi* in camels, horses and donkeys.

Figure 1E: Quinapyramine/Antricyde
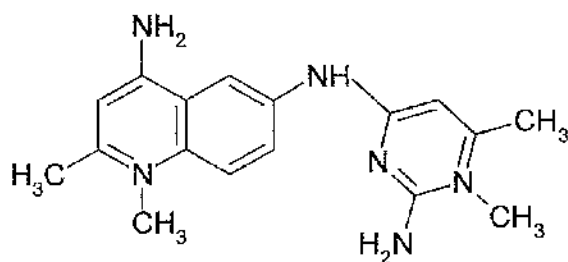
The Emergence of Drug Resistance

The most important problem associated with current chemotherapeutic drugs is the emergence of drug resistance, which, with the low level of screening, is largely responsible for the recent re-emergence of sleeping sickness. Resistance, to different extents, has now been reported to every veterinary trypanocidal drug and also to some drugs available for the treatment of HAT. The resistance is rapidly increasing and spreading throughout the African continent and as reports are mainly obtained from small-scale studies the extent of the problem may be grossly underestimated. Drug resistance obviously renders infected people and cattle refractory to treatment and the problem is amplified due to the lack of the development of new drugs at present or in the foreseeable future.

It is thought that resistance to a drug takes about ten years to develop. Resistance can occur in two ways, where individuals in the population with genes conferring a drug resistant phenotype as opposed to a drug sensitive one, are selected for in response to drug pressure. However, the preferred theory is that the parasite becomes genetically altered in some way through an adaptive process similar to that of antigenic variation. This is known as "acquired" drug resistance. There are several factors thought to be responsible for the induction of drug resistance. One of the most important is exposing the pathogen to sub-lethal doses of a drug, which allows it to continue to divide and replicate in the presence of the drug, encouraging selective mutations to arise. Treating an infected patient with a sub-therapeutic dose or by using a drug with a long half-life that is slowly eliminated from the body of the host, aids this process. Often compliance of patients to complete a course of treatment is low due to adverse side effects and invasive diagnostic procedures, which will ultimately lead to cases of relapse. As many trypanocides have an affinity for DNA and may therefore be mutagenic, they themselves may cause mutations to produce a resistant phenotype (Geerts and Holmes, 1998).

There is also evidence to suggest a positive correlation between high tsetse fly challenge and an increase in the level of drug resistance in a particular area. This is probably due to an increase in drug use in these areas (Geerts et al, 2001).

At a molecular level, several biological modifications in a parasite could be responsible for a resistant phenotype. Changes in membrane permeability or a reduction in specific membrane receptors or transporters could lead to a reduction in uptake of the drug preventing it from reaching toxic levels or the target in the cell (Sutherland and Holmes, 1993). In some cases the receptor or transporter may be lost completely, preventing drug

access into the cell. In other cases an up-regulation of drug efflux pumps can occur, again to prevent the drug reaching toxic levels in the cell. This is found to be a common cause of multi-drug resistance. P-glycoproteins are a set of energy-dependant efflux pumps encoded by a multigene family that shares a considerable amount of homology with bacterial membrane associated proteins. They are part of a class of ABC transporters which couple ATP hydrolysis to transport substances across the cell surface membrane (Matovu et al, 2001). Over-expression of these pumps in response to drug pressure is responsible for increased efflux of the drug and the development of drug resistance. Agents that block these efflux pumps can often reverse this type of drug resistance (Hayes and Wolf, 1990, Dalton et al, 1989). These agents, for example calcium antagonist verapamil are unable to reverse resistance to trypanocides and therefore the mechanisms of drug resistance in trypanosomes do not appear to be the same as those for *Plasmodium* or cancer cells (Anene et al, 1995, Fairlamb et al, 1992). However, recently three genes encoding ABC like transporters have been identified in *T.brucei*, but their function is unknown (Mäser and Kaminsky, 1998). A change in the intracellular target can also cause reduced affinity of the drug for the ligand. It is unclear whether drug-resistant trypanosomes are less pathogenic, which has been suggested for at least one laboratory strain (Berger et al, 1995). A mutation relating to drug resistance may affect virulence factors, which may lead to a reduction in pathogenicity. However, this is a controversial issue and has been disputed (Ainanshe et al, 1992, Geerts et al, 2001).

Solutions to delay drug resistance.

In humans, it is best that the patient remains in hospital until the infection has cleared to prevent vector transmission of drug resistant parasites. In cattle, it is important to avoid the administration of sub-therapeutic doses. This is often a problem as farmers may underestimate the body weight of their animals. Also, unskilled workers are allowed to administer the drugs, which are often diluted to save money and there is a constant increase of poor quality drugs leaking onto the market that do not contain the stated amount of active drug ingredients. This could be solved by quality control and release of drugs as a single dose treatment. The use of sanative pairs, such as isometamidium and Berenil, can reduce the development of resistance as there is little cross resistance between them and because both drugs have separate targets, it is unlikely a parasite will evolve a mutation converging resistance to both drugs. It has been found, however, that the use of Berenil with either isometamidium or homidium leads to a higher rate of mortality than if the latter two drugs are used independently (Dolan et al, 1992). The number of drug treatments can be reduced by using an integrated control programme. This is particularly important in areas of high tsetse fly challenge where the number of drug treatments will be increased, in turn leading to an increase in selective drug pressure. The increase in number of drug treatments also poses a threat to the environment due to the excretion of contaminants (Anene et al, 2001). Mass treatments

should also be avoided at all costs and individual cases should be treated. Once resistance to a drug has transpired the dose of the drug should not be increased as this will lead to a higher level of resistance. It is better to remove the drug from the market and allow the resistant phenotype to revert back to the sensitive wild type profile. However, the best way to prevent drug resistance in the first place is to administer high doses of drugs that will kill all residing parasitaemia in the host. Unfortunately, all trypanocidal drugs are toxic in high doses and the margin of safety between cure and toxicity in extremely narrow (Anene et al, 2001). In the case of multiple drug resistance, chemotherapy is useless and the only feasible option is through vector control (Geerts and Holmes, 1998).

It is imperative that we gain an accurate insight into the extent of drug resistance currently present in the population to allow appropriate control measures to be applied to different geographical areas. There are several ways to test trypanosomes for drug sensitivity and monitor the type of infection present in cattle. Small ruminants can be used to assess the concentration of trypanocide required to produce either a permanent cure or to clear all parasites from the blood. However, these tests are not only time consuming but are also expensive. Cattle must also be kept in fly-proof sheds throughout the test to prevent re-infection, which may not always be possible. As an alternative, tests can be carried out on mice, which is relatively successful for *T.brucei brucei. In vitro* assays have the biggest advantage allowing for large numbers of field isolates to be tested simultaneously and if metacyclic organisms are used, the results can be applied directly to the field. However, assays can also prove to be expensive (Hirumi et al, 1993), not all types of trypanosomes grow well *in vitro* (e.g. *T.b.gambiense* and *T.congolense*) and phenotypes may change during adaptation to the new conditions.

Resistance to Veterinary Drugs

Resistance to diminazene, homidium and isometamidium has now been reported in 11 countries throughout sub-Saharan Africa (Peregrine, 1994). Resistance to Berenil by a *T.congolense* strain in Nigeria was first reported in the field in 1967 (MacLennan and Jones-Davies, 1967) It has now been repeatedly documented in Nigeria (Jones-Davies, 1968) Chad, Kenya, Tanzania, Somalia, Burkina Faso, Uganda and Ethiopia. There is also evidence to support long-term resistance to berenil, shown in the Ghibe Valley, Ethiopia, where berenil resistance was first described in the 1980s and is still highly prevalent today (Rowlands et al, 2001, Rowlands et al, 1993). Resistance to Berenil can develop in three ways: through repeated exposure to sub-lethal doses, the development of cross-resistance with quinapyramine or innately, with no previous exposure. However, although resistance to this drug has been reported the incidence is relatively low compared to resistance levels to the other veterinary and arsenical trypanocides. Widespread and long-term use does not ultimately lead to the development of resistance and induction of resistance in the laboratory by exposing infected mice to sub-curative doses of Berenil has been virtually impossible (Whiteside, 1963). In one study, even after 40 months of drug exposure the resistance to Berenil was still low (Peregrine and Mamman, 1993, Hawking, 1963a). This may be attributed to the fact that in Berenil resistant populations the number of parasites actually resistant to the drug is thought to be

as low as 1 in 10^3 parasites (Mamman et al, 1995b). In the field, perhaps low resistance levels are due to the induction of a regime that involves the use of sanative pairs in endemic areas. In 1983 Njou reported a strain of *T.congolense* that was resistant to 7mg/kg of the drug, which is double the recommended dose. However, the infection could be cleared by ISMM, the drug's sanative pair (Mbwambo et al, 1988). This is often found to be the case (Desquesnes et al, 1995) and this is the recommended course of action in Berenil resistant areas.

Low resistance may also be attributed to the rapid elimination of the drug from the body of the host. On the other hand, resistant isolates appear to retain their resistant phenotype after several passages of cyclical transmission and therefore once resistance to Berenil occurs it remains stable (Gray and Roberts, 1971). Relapsing infection is often taken to be a sign of drug resistance. However, it has been found that if trypanosome strain from a relapsing host is inoculated into a new host, treatment with Berenil clears infection if administered within 3 days of reinfection. If left for a longer duration Berenil is ineffective possibly due to the parasites being relocated in an inaccessible area, for example cryptic sites (Jennings et al, 1977, Mamman et al, 1993). The dose for clearing relapse infections is affected by the initial size of inoculum of parasites. As the number of parasites in the inoculum increases the dose of drug required to clear infection also increases. This will affect the level of resistance in the population (Mamman et al, 1995a). The dose at which relapse occurs can also be enhanced by gradually exposing the resistant parasites to increasing levels of Berenil (MacLennan and Na'isa, 1970).

T.vivax populations have been shown to express high levels of resistance to the prophylactic action of ISMM but not to the therapeutic action (Peregrine et al, 1991a) In 1991, Sutherland et al showed that resistance to ISMM was associated with reduced accumulation of the drug, which was demonstrated by a reduction in fluorescence of the drug. Lysis of the cells produced a higher fluorescence and therefore an increase in drug uptake, which showed the involvement of the cell membranes in limiting drug uptake in resistant clones. It was later found that ISMM is transported across the cell membrane via a protein carrier (Zilberstein et al, 1993). Accumulation of the drug within the cell involves a specific receptor located on the surface membrane. The addition of calcium antagonists increased the accumulation of ISMM in resistant clones indicating that an efflux mechanism is involved in the development of resistance to this drug and this mechanism is energy dependent (Sutherland et al, 1992a). This was supported through the use of a kinetic model to determine the cause of reduced accumulation (Sutherland et al, 1992b). It has also been found that sensitive clones accumulate most of the drug at pH 7.4 whereas the optimum pH for accumulation in resistant clones is pH7, which indicates a change in receptors (Sutherland and Holmes, 1993). It has also been suggested that the modulation of the mitochondrial potential is involved with development of resistance to ISMM. When agents were used to disrupt mitochondrial function the mitochondrial membrane potential was inhibited along with the inhibition of isometamidium uptake and accumulation (Wilkes et al, 1997). The number of ISMM transporters in the plasma membrane of resistant strains also appear to be reduced demonstrated by a lower V_{max} but an equal K_m value to the sensitive strains.

Homidium appears to be worst affected by drug resistance and is now basically rendered useless as an effective treatment. Williamson and Stephen first recorded resistance to the drug in 1960 and it appears that most relapses are due to *T.congolense* infection. By 1965 resistance was widespread and since then there have been continued reports (Scott and Pegram, 1974). It has been demonstrated that animals infected with drug resistant trypanosome strains eliminate homidium more rapidly from their bodies but the reason for this is unclear (Murilla et al, 2002).

Cross-resistance is often observed between homidium and isometamidium (Schönefield et al, 1987). In this case Berenil is the recommended treatment as no cross-resistance has been observed between ISMM and Berenil, although sometimes repeated doses are required to obtain a complete cure (Küpper and Wolters, 1983). Surprisingly, there appeared to be cross-resistance to Berenil and homidium in 1968 when there had been none the year previously (Jones-Davies, 1968, Na'isa, 1967). Berenil and homidium have both been shown to interact with kinetoplastid DNA to produce dyskinetoplastids. As the kinetoplast is apparently the drug target for both drugs, the loss of this organelle will lead to resistance to these drugs. However, this appears to be an isolated case of cross-resistance and was never proved to exist through laboratory techniques. In areas where multiple drug resistance has not been detected Berenil can be used to treat homidium resistant cases, as generally there appears to be no cross-resistance between them (Gadir et al, 1972).

The drug quinapyramine, the original prophylactic agent, which was withdrawn from the market in 1976, was not only removed due to the development of resistance to it, in particular, but because it appeared to be associated with multiple drug resistance to Berenil, isometamidium and homidium. This cross-resistance is extremely easy to induce in the laboratory (Ndoutamia et al, 1993). Multiple resistance to all three drugs by isolated *T.congolense* clones has now been reported in the Ghibe Valley, Ethiopia, (Afewerk et al, 2000, Mulugeta et al, 1997, Codjia et al, 1993) Burkina Faso (Clausen et al, 1992) Southern Somalia (Ainanshe et al, 1992) and Sudan (Mohamed-Ahmed, 1992). In these areas sanative pairs diminazene and isometamidium were used and both drugs failed to cure infected animals at the normal dose rate. It is more worrying that resistance has developed to both these drugs because there is no chemotherapeutic alternative left. However, although trypanosome strains can develop resistance to both berenil and ISMM (Peregrine et al, 1991b, Chitambo and Arakawa, 1991, Moloo and Kutuza, 1990) this resistance to each, occurs independently and as yet there has been no cross-resistance demonstrated between the two drugs. This at least is good news that the use of one drug will not automatically lead to the development of resistance to the other (Chitambo and Arakawa, 1992).

Transporters of *T.brucei*

The main difference between the metabolism of trypanosomes and their host is that trypanosomes are unable to synthesise purines *de novo* and as a result must scavenge them from the host environment. This is achieved through the transport of the purines

into the cell via surface membrane transport systems. The uptake of substances can either be passive by diffusion or through active transport, the latter involving the accumulation of a substrate to high concentrations within the cell. It is thought that there are at least 400 genes for transport proteins in the *T.brucei* genome and a large amount of these will be expressed on the surface of cells for the transport of substances (Borst and Fairlamb, 1998). It is important to understand the mechanisms of purine uptake in these organisms to allow the generation of trypanocides that can utilise this transport system and be delivered into the cell. In 1980 James and Born showed that *T.brucei* and *T.congolense* consumed adenosine and to a lesser extent inosine and guanosine from the surrounding environment. This transport was saturable and conformed to Michaelis-Mentin kinetics. It was suggested that the tissue damage inflicted upon the host by the parasite increased the concentration of amino-purines in the extracellular fluid. Adenosine is a ribonucleoside found either freely in cells or as part of nucleic acids. It can inhibit or stimulate the release of neurotransmitters and is also involved in platelet and neutrophil functions. (Plagemann et al, 1988) In excess it can cause marked cardiovascular effects (Griffith and Jarvis, 1996). Inosine is a hypoxanthine riboside found in meat and yeast. It is a product of the first stage of the breakdown of adenosine to uric acid (Wang, 1995). Bloodstream forms of *T.brucei* have been shown to posses several transporters for the uptake of nucleosides and nucleobases. (Listed below).

Transporter	Substrate transported into the cell
P1	Inosine, Guanosine, Adenosine
P2	Adenosine, Adenine, Melaminophenyl arsenicals, Pentamidine
H2	Hypoxanthine, Adenine, Guanine
H3	Hypoxanthine, Adenine, Guanine
HAPT1	Pentamidine and Propamidine
LAPT1	Pentamidine and Propamidine

Two hypoxanthine transporters have been characterised in bloodstream forms, H2 and H3. They have specificities for purine/pyrimidine bases and guanosine and purine bases, respectively. They have no affinity for adenosine (De Koning and Jarvis, 1997). *T.brucei* also possesses glucose transporters for the uptake of its main energy source, glucose (Borst and Fairlamb, 1998). Most importantly, two adenosine sensitive transporters have been identified (Carter and Fairlamb, 1993, De Koning and Jarvis, 1999). The P1 transporter mediates the transport of adenosine, inosine and guanosine. The P2 transporter is specific for adenosine and adenine. The P1 transporter has a higher affinity for adenosine than the P2 transporter with Km values of $0.35\mu M$ and $0.94\mu M$, respectively (De Koning and Jarvis, 1999). An adenosine transporter has also been identified in procyclic *T.brucei* that appears to function as an H^+ symporter and is identical in specificity to the P1 transporter found in bloodstream forms (De Koning et al, 1998). The insect-dwelling form also has an H1 hypoxanthine transporter. These types of

nucleoside/nucleobase transporters are present in a variety of other organisms. There are two nucleoside transporters in mammalian cells designated N1 and N2 which have similar substrate specificities to the P1 and P2 transporters (Griffith and Jarvis, 1996). *Leishmania donovani* has also evolved to possess two high affinity nucleoside transporters to scavenge nucleoside substrates from the environment, which is an effective system even when the concentrations of nucleosides available are extremely low. The specificities for these transporters are independent of each other and therefore the two transporters can be distinguished structurally and kinetically. The first has affinity for inosine and guanosine and the second for adenosine, adenosine analogues and pyrimidines. Deficiencies in the uptake of these substances can be attributed to two mutations (Aronow et al, 1987).

T.cruzi also has two nucleoside transporters, which are specific for purines and pyrimidines, respectively (Finley et al, 1988).

The role of nucleoside transporters in drug resistance

In HAT the major concern is the rapidly increasing resistance to melarsoprol, as this is the only effective and widely available drug used to treat late stage sleeping sickness. Currently, up to 20% of people in areas of epidemic are not responding to treatment with the drug (Barrett, 1999). DFMO has been implemented as a backup drug used to treat patients who are unresponsive to melarsoprol but it is expensive. Nufurtimox has also proved successful in the treatment of melarsoprol relapse patients although has a plethora of associated side effects.

In 1959, Williamson discovered that the lysis of melamine sensitive trypanosomes was inhibited by the amino-purines adenine and adenosine. This showed that the melaminophenyl arsenicals were competing for the same transport system as amino-purines. The P2 transporter was identified as the carrier for melaminophenyl arsenicals. A drug efflux mechanism is not involved in the development of resistance (Bacchi, 1993, Carter and Fairlamb, 1993).

Arsenical sensitive *T.brucei* readily absorbs and concentrates arsenical compounds whereas resistant lines do not. An arsenical resistant *T.brucei* line, RU15, has reduced adenosine uptake with a 3-fold reduction of V_{max} for the P1 transporter and P2 activity cannot be detected This suggests that the melaminophenyl arsenical resistant phenotype is associated with modulated P2 transporter activity (Carter and Fairlamb, 1993). The mutation that caused melarsoprol resistance was reversible and P2 activity could be restored. Defective P2 function confers melarsoprol resistance (De Koning et al, 2000). The salvage of purines is not critically affected by the loss of P2 transporter function because the P1, H2 and H3 transporters can still transport sufficient amounts of purines into the cell.

Since 1951 it has been noticed that clinical isolates from patients refractory to melarsoprol treatment and laboratory produced arsenical resistant strains presented cross-resistance between melamine based arsenicals (melarsen) and diamidine compounds (pentamidine) (Rollo and Williamson, 1951, Williamson, 1951). This cross-resistance is present in both *T.b.rhodesiense* and *T.b.brucei* isolates. Cross-resistance usually suggests that the drugs have a common drug target or a common uptake mechanism. It is unlikely that diamidines and arsenicals have the same trypanocidal mode of action because

induced cell death by arsenicals is extremely rapid whereas diamidines must accumulate to higher levels in the cell before lysis occurs (Barrett and Fairlamb, 1999). There is a correlation between cross-resistance and the interatomic distances between the benzamidine rings. The greater the distance between the rings in a particular diamidine compound, the lower the cross resistance with melarsoprol (Fairlamb et al, 1992).

In 1975 Damper and Patton identified a transport system for pentamidine in *T.brucei* that was saturable and conformed to Michaelis–Menten kinetics. It could be competitively inhibited by other aromatic diamidine compounds such as propamidine, hydroxystillbamidine and benzamidine (Damper and Patton, 1976a, 1976b). Drug resistant strains were found to accumulate less pentamidine than drug sensitive strains suggesting that resistance is related to a reduction in drug accumulation. The integrity of the cell membrane is important in the development of diamidine resistance because the use of Triton X to break down the surface membrane leads to the accumulation of drugs by the resistant trypanosomes (Sutherland et al, 1992a).

In 1995 it was demonstrated that pentamidine and other aromatic diamidines were substrates for the P2 transporter (Carter et al, 1995). Adenosine transport by P2 is dose-dependently inhibited, by 95%, as concentrations of pentamidine increase. Melarsen resistant cells had a reduced ability to transport adenosine and pentamidine. The resistant strain RU15 also had a reduced capacity and affinity for the uptake of pentamidine. However, it has also been shown with other resistant strains that the accumulation of pentamidine is unaffected and that reduced drug uptake is not the cause for drug resistance (Berger et al, 1995).

The P2 transporter recognises a common motif that is found on amino-purines, melaminophenyl arsenicals and diamidine compounds (Figure 1F). This supports Paul Erlichs theory in 1898 that a drug is comprised of a haptophoric part that is recognised by a cell receptor and a toxophoric part that administers the pharmacological action. When resistance to aromatic arsenicals develops the resistance is directed towards the non-arsenical part of the drug, or the haptophoric part (Williamson and Rollo, 1959). Therefore, this can be exploited by designing drugs that present this motif attached to a toxic compound. The drug can then be recognised by the transporter and will be taken into the cell where the toxic part of the molecule can work on a drug target. This has been successful where polyamine analogues, which compete with enzymes in the polyamine

Pentamidine   Melarsoprol
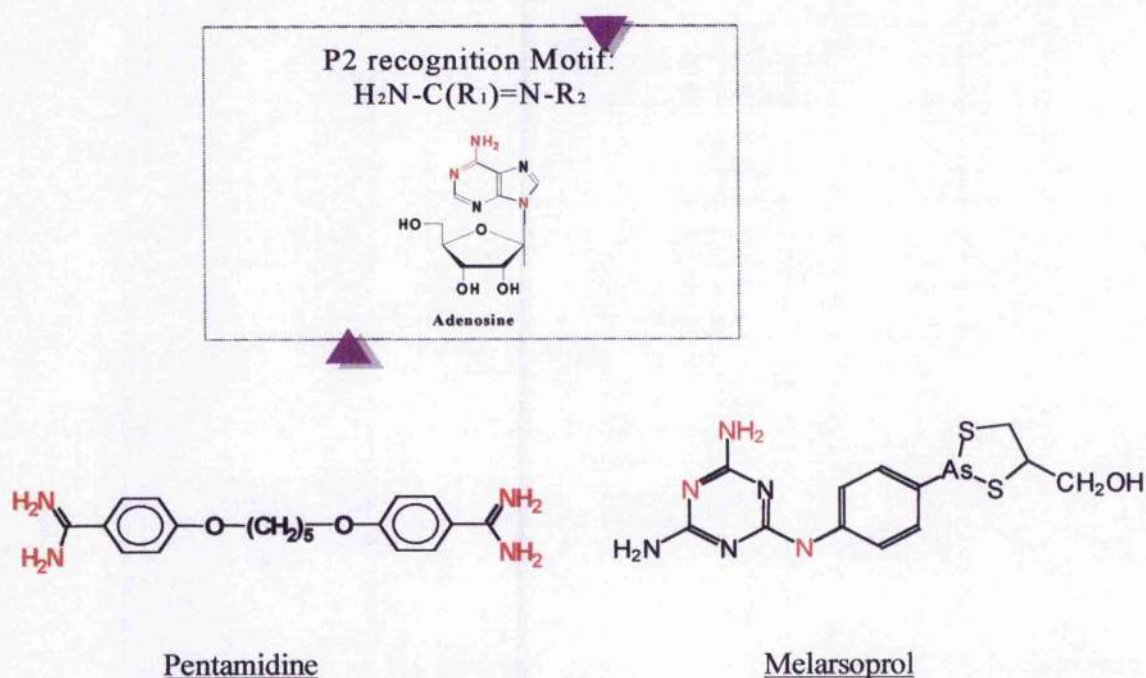
Figure 1F: The P2 recognition motif is present on adenosine, arsenicals and aromatic diamidines and is indicated in red.

biosynthetic pathway, were coupled to the P2 recognition motif (Tye et al, 1998). The ribose moiety is not necessary for ligand binding to the P2 transporter. However, the purine ring is extremely important, the crucial part being the amine group at position 6 on the ring. Compounds containing this motif generally have high affinity for the P2 transporter, but the motif alone does not guarantee binding. Binding energies of melarsoprol and pentamidine, which both contain this motif, are high. However, the binding energy of Berenil is much lower probably due its more rigid structure caused by the double bond in the centre of the molecule (De Koning and Jarvis, 1999).

Recent studies showed that by cloning the gene TbAT1 (*T.brucei* adenosine transporter 1) in the yeast *Saccharomyces cerevisiae* led to the identification of a P2 type of transporter with an affinity for adenosine. Yeast expressing this transporter was also sensitive to melaminophenyl arsenicals. Substrate specificity and transport kinetics appeared to correspond to the P2 transporter. However, the cells were insensitive to the diamidine compounds (Mäser et al, 1999). By comparing the cDNA sequence for *TbAT1* of wild-type *T.brucei* and arsenical-resistant *T.brucei*, a change in six amino acids was observed. Therefore individual strains are producing two forms of the same molecule on the surface. This modulation can be induced by exposing the parasites to selective drug pressure in the laboratory and has also been identified in a strain isolated from a relapse patient (Kaminsky and Mäser, 2000). Therefore this gene can be used to distinguish between sensitive and resistant trypanosomes (Matovu et al, 2001). This marker for resistance could be useful as a diagnostic technique in the field. *TbAT1* gene shows considerable homology to the human nucleoside transporter *hENT1* and *hENT2* as well as

to *L.donovani* transporters LdNT1 and LdNT2 (Carter et al, 1999). A P1 transporter gene TbNT2 from *T.brucei* has also been cloned and when expressed in *Xenopus* oocytes it leads to the uptake of inosine, guanosine and adenosine. There are six other genes present in this cluster, which may code for other transporters and are therefore possibly involved in drug resistance (Landfear, 2001, Sanchez et al, 1999).

Although there is widespread arsenical resistance in the field, the drugs used to treat early stage HAT (Suramin and Pentamidine) do not appear to be affected by the threat of drug resistance and are still reliable at producing an acceptable cure rate (Kaminsky and Mäser, 2000). If both arsenicals and pentamidine are solely substrates for the P2 transporter and loss of this protein leads to arsenical resistance it would be expected that cross-resistance to pentamidine would also occur. In studies, which show cross-resistance between arsenicals and diamidines, there is always an alteration in activity of the P2 adenosine transporter. However, lines resistant to arsenicals have been shown to be sensitive to pentamidine, which led to the idea that there must be more than one route of entry for pentamidine into the cell (Barrett and Fairlamb, 1999). In sensitive cells, [^{3}H]-adenosine transport is inhibited by pentamidine and [^{3}H]-Pentamidine transport is inhibited by adenine and melaminophenyl arsenicals but surprisingly is only partially inhibited by adenosine, again suggesting another route of pentamidine into the cell (Carter et al, 1995). As previously mentioned strain RU15 appeared to have a loss of P2 function and although resistance occurred to arsenicals there was only very minor resistance to pentamidine. Also, there is only ever reduced uptake of pentamidine in resistant cells and not a complete lack of uptake and therefore it is likely that pentamidine

46

enters the cell by more than one transporter. This was found to be the case. Pentamidine is taken up by multiple transporters. An adenosine –sensitive pentamidine transporter (ASPT1) which has all the characteristics of a P2 transporter, a high affinity pentamidine transporter (HAPT1) with a K_m of 36 nM and a low affinity pentamidine transporter (LAPT1) with a K_m of 56 µM (De Koning and Jarvis, 2001, De Koning, 2001). The fact that there are three transporters could explain the lack of cross-resistance between arsenicals and pentamidine in the field. If P2 is lost through selective drug pressure the drug will still be able to penetrate the cell through HAPT1 and LAPT1. Both of these transporters are retained in a *T.brucei* line, Δ*TbAT1*, in which both copies of *TbAT1* had been deleted (Matavu et al, unpublished). However, the true biological function of these transporters is unknown. It is important to discover the natural substrates for HAPT1 and LAPT1 and study the structural and binding relationships between substrates and the transporters because drugs can then be designed with specific affinity to these carriers. This would be extremely useful in the field where P2 was lost through selective drug pressure because drugs could still be targeted to trypanosomes through these other entry sites. I therefore looked at the structural relationships between HAPT1 and LAPT1 and various trypanocides and diamidine analogues in an attempt to identify the motifs crucial for uptake by these transporters. This was carried out using unlabelled test compounds to inhibit the uptake of [^{3}H] pentamidine.

Tranporters and diminazene resistance

A high level of cross-resistance has been observed between melarsoprol and the veterinary diamidine drug berenil. This is unusual because cross-resistance between melarsoprol and pentamidine, in the field, is low. It is likely that Berenil is taken up by one transporter only and that the loss of this transporter confers resistance. It is also likely that berenil enters the cell via the P2 transporter, which would explain the high level of cross-resistance between melarsoprol and Berenil. Studies on *T.evansi* showed that adenosine transport by the P2 transporter was dose-dependently inhibited by Berenil. This is not conclusive evidence that Berenil is transported by the P2 transporter, but it suggests that this is probably the case. However, when Berenil was used in combination with adenine to inhibit adenosine transport by P2, there was a moderate, but not significant, increase in the inhibitory effect. This could suggest the presence of another Berenil transporter (Suswam et al, 2001), but the data are inconclusive.

Alterations in the P2 nucleoside transporter have been associated with reduced uptake of diminazene aceturate. Studies with a Berenil resistant *T.equiperdum* clone revealed that the P2 transporter had a significantly reduced affinity for adenosine although the transporter appears to be retained. Therefore, perhaps the P2 transporter is actually lost which allows the presence of a low affinity adenosine transporter with a similar substrate specificity to be detected. However, it appears that Berenil resistance is related to a change in function of the P2 transporter (Barrett et al, 1995). Arsenical resistant clone RU15 displays strong cross-resistance with berenil but not with diamidine pentamidine

(Fairlamb et al, 1992). This can be explained by the presence of the two additional pentamidine transporters HAPT1 and LAPT1.

Recently a TbAT1 null mutant has been constructed and was found to be only partially resistant to melaminophenyl arsenicals and pentamidine but showed a very high level of resistance to Berenil. This again supports the idea that berenil enters the cell via the P2 transporter only. Melarsan oxide appeared to be entering through both P2 and HAPT1. This would suggest that arsenical resistance requires the loss of both of these transporters (Unpublished).

If the sole transporter for diamidine compounds is the P2 transporter, then there should be a high incidence of Berenil resistance in the field. In fact, there is quite a lot of resistance, but perhaps lower than might be expected. This is mainly the result of the pharmacokinetics of diminazene being cleared quickly so it does not linger at a subcurative level strong enough to induce selection of resistance. I conducted a series of experiments to identify the P2 transporter as the mediator of Berenil uptake and detect the presence of any other Berenil transporters.

Crithidia fasciculata

Crithidia fasciculata is a monoxenous flagellated trypanosomatid of mosquitoes. The organism has been of biochemical interest since 1933 when Margeurite Lwoff showed that the parasite could be grown easily in peptone water by adding a little haem or blood (Kidder and Dutta, 1958). Different *Crithidia* species have been used as models to study

biochemical pathways and gene expression of trypanosomes, as the two organisms share common biochemical features. Two high affinity purine nucleoside transporters have been identified in *C.fasciculata* . CfNT1 is an adenosine transporter with a K_m of 9.4 ± 2.8 μM for the substrate. CfNT2 demonstrates mediated uptake of inosine with a K_m of 0.38 ± 0.06 μM and also recognises guanosine but not adenosine. (De Koning et al, 2000b). The uptake of these substrates is saturable and conforms to Michaelis–Menten kinetics. The specificities of these transporters loosely resemble those of P1 and P2, identified in *T.brucei brucei*. However, in this case, the stage of life cycle affects purine uptake, for example, during logarithmic phase the uptake of adenosine and inosine is dramatically increased (De Koning et al, 2000b). Two purine transporters with identical substrate specificities to CfNT1 and CfNT2 have also been identified in *C.luciliae* (Hall et al, 1996).

HAPT1 and LAPT1 of *T.brucei brucei*, which have been previously discussed, both recognise the trypanocidal drug pentamidine but the true biological substrate for these transporters is unknown. If these transporters can be expressed in another trypanosomatid, which lacks a pentamidine transporter, the sequence responsible can be isolated and compared to other transporter sequences that will perhaps allow us to characterise LAPT1 and HAPT1 and ultimately discover their biological function. *C.fasciculata* has been chosen as the organism to act as an expression system in this study. There are several reasons for this. *Crithidia* grows extremely fast, reaching maximum density of 2×10^8 organisms/ml in 48 hours, it can be grown easily on chemically defined media and a relatively large amount of work has also been carried out involving the uptake and effects of pentamidine. In 1966, Gutteridge added pentamidine

to cultures of *C.fasciculata* during exponential phase and found that the net synthesis of protein, RNA, DNA and phospholipid was decreased. At 500 μM glucose stimulated respiration was also found to be sensitive to the drug. Resistance to pentamidine can be induced by exposing the organisms to increasing concentrations over a period of about 5 months, which is relatively slowly compared to the development of resistance against arsenicals (Wallis 1966), (This is similar to the situation in the field with development of drug resistance in trypanosomes). A resistant line was able to grow in pentamidine at a concentration of 500 μM and this was maintained for a period of 1 year and 7 months. However, after this time, drug resistance fell away rapidly. In another study, involving pentamidine resistance in *C.oncopelti*, cross resistance between other diamidine groups and arsenicals was demonstrated and it was suggested that diamidine resistance may be due to the inability of the drugs to reach there normal drug targets within the cell. (Wallis 1966).

Although a specific pentamidine transporter has not been identified in *C.fasciculata* the organism is obviously taking up the drug and this can be prevented by induction of pentamidine resistance. Perhaps the drug is being taken up by CfNT1, which would be a similar situation to the uptake of pentamidine, by the P2 transporter in trypanosomes.

It is therefore important to determine the absence or presence of a pentamidine transporter in the organism of choice, *C.fasciculata* strain HS-6. If the results are negative for mediated pentamidine uptake, the organism is ready to be used as an expression system. If a pentamidine transporter is discovered this must be eliminated through the induction of drug resistance, where presumably the transporter will be lost. Verification that resistance is caused by loss of pentamidine transport will be required. By

transforming a DNA library into the organisms lacking a pentamidine transport system, cells expressing either HAPT1 or LAPT1 will be those able to take up pentamidine. HAPT1 and LAPT1 will be identified by their distinct substrate affinities and specificities.

Chapter 2

Materials and Methods

Trypanosomes

Bloodstream forms of *Trypanosoma brucei*, strains S427 (wild type) or *T. brucei* lacking the TbAT1 adenosine transporter gene (Δ TbAT1) were taken from frozen stocks and used to infect female Wistar rats via intra-peritoneal injection. Following a 48-hour incubation period, the blood was removed under anaesthetic at the point of peak parasitaemia by cardiac puncture, using heparin as an anti-coagulant. The blood was kept on ice before being centrifuged at 2500 rpm for 15 minutes. This allows the separation of the blood into three different components: a red blood cell layer, a plasma layer and a white buffy-coat layer which is found between the former two and contains the trypanosomes. This layer was gently removed using a pasteur pipette and resuspended in a phosphate saline glucose solution (PSG) (51.84 mM Na_2HPO_4, 3.29 mM $NaH2\ PO_4$, 72.7 mM NaCl and 61mM glucose in ddd H_2O, pH 8). The trypanosomes were isolated from the remaining red blood cells by passing the buffy coat layer through a DE52 cellulose Whatman anion exchange column. (Lanham 1968) Trypanosomes are more positively charged than rat red blood cells between the pH range of 6-9 and therefore the red blood cells become trapped in the cellulose whilst the trypanosomes are allowed to pass through. DEAE-cellulose was added to the column to a depth of roughly 5cm. Alternatively, a 50ml syringe can be used with a small amount of glass wool placed in the bottom. The column was washed through with 200 mls of PSG, pH8. It is important that the pH of the column is exactly 8.0 otherwise the blood cells will run through. The cellulose was stirred and left to settle and the buffy-coat layer was loaded on top. PSG was used to wash the trypanosomes through the column which were collected and then washed twice in assay buffer (14 mM glucose, 33.6 mM

HEPES, 23.9 mM MOPS, 23.8 mM $NaHCO_3$, 4.7 mM KCl, 0.3 mM $MgCl$, 97.5 mM NaCl, 0.08 mM NaH_2PO_4, 2.8 mM $CaCl_2$, 0.08 mM $MgSO_4$, pH 7.3) by centrifugation at 2200 rpm for ten minutes at 4°C. A cell count was performed using a haemocytometer and the cells were resuspended in the required volume to produce the desired cell number of 1 x 10^8/ml. The cells were left at room temperature for 20 minutes to allow them to adapt to the experimental conditions become more active for drug uptake. Motility of the cells was observed using a microscope at the end of the experiment to ensure the cells were viable throughout. All contaminated materials were disposed of using bleach.

Crithidia fasciculata

C. fasciculata strain HS-6 which was isolated by Shim and Fairlamb (1988) was used in transport studies and drug sensitivity assays. The organisms were cultivated in LIT medium (Liver Infusion Broth 9 g/litre, Tryptose 5 g/litre , 20 mM NaCl, 56 mM NaH_2PO_4, 5.3 mM KCl, 5.5 mM Glucose, Haemin 10mg/litre, pH 7.2) which is autoclaved for 15 minutes at 15 psi before adding 10 % Heat Inactivated Foetal Calf Serum and Gentamicin sulphate at 25 $\mu g.ml^{-1}$. The cultures were maintained at 25°C and were passaged every 2-3 days into 10 mls of fresh medium. For transport assays 2 mls of culture in logarithmic growth phase (1-2 x 10^7 cells/ml) were added to 100 mls of fresh medium and incubated for about 24 hours. The cells were harvested by centrifugation at 2200 rpm for 15 minutes and then washed twice in assay buffer. Normal practise is to keep cells at 4°C during this process. However, this is not necessary for _Crithidia_ and it is acceptable to centrifuge cells at 25°C. A cell count was carried out using a haemocytometer and the cells were resuspended in the required volume of assay buffer to give 1 x 10^8 cells/ml. For drug sensitivity assays the final concentration of cells should be 5 x 10^4 cells/ml and they do not have to be transferred

to assay buffer. It is important to keep cells under sterile conditions during passage to avoid contamination of cultures.

Radiolabeled compounds, diamidine analogues and other test compounds.

[^{3}H] Pentamidine isethionate (3.63 TBq/mmol) and [^{3}H] Diminazene aceturate (3.07 TBq/mmol) were synthesised by Amersham Pharmacia Biotech for Dr M.P. Barrett and Dr H. P. de Koning (University of Glasgow), respectively. Both radiolabeled compounds were stored at -20°C. The test compounds were either purchased from Sigma (adenosine, benzamidine, diminazene, DAPI, inosine and ethidium bromide) or Rhône-Poulenc in France (pentamidine, stillbamidine, propamidine isethionate, hexamidine, heptamidine and octamidine) with the exceptions of butamidine which was generously donated by Dr Alan Fairlamb (University of Dundee). The diamidine analogues, meta-pentamidine and ethamidine were sent from Professor Richard Tidwells' laboratory at the University of North Carolina and the DWB compounds 075, 569 and 544 were synthesised by Dr David Boykin at the University of Georgia State who also works in collaboration with Professor Tidwell.

Uptake Assays for Transport Studies

The uptake of radiolabeled [^{3}H] Pentamidine and [^{3}H] Diminazene aceturate were determined using the method described previously (De Koning and Jarvis 1997, De Koning et al. 1998) with appropriate modifications for this study.

Test compounds

Stock solutions of test compounds were made in assay buffer to the highest concentration possible, which was determined by the solubility properties of each compound. For example Pentamidine can reach up to 10mM in assay buffer whereas Diminazene only reaches about 4mM. Due to insolubility it was not always possible to obtain the concentration required for full saturation of the transporter and therefore several saturation curves are incomplete. A series of dilutions were made over a decreasing concentration gradient of wide range. The concentrations should be four times the desired final concentration.

Radiolabeled permeant

The [^{3}H] Pentamidine and [^{3}H] Diminazene aceturate were made up in assay buffer to four times the desired final concentration. The concentration of the permeant was selected several-fold below the K_m of the transporter being studied. The High Affinity Pentamidine Transporter (HAPT1) has high binding affinity for pentamidine and therefore the transporter becomes saturated at a low drug concentration requiring the final concentration in the assay to be 10 nM. The Low Affinity Pentamidine Transporter (LAPT), however, has lower binding affinity for the drug and necessitates the higher permeant concentration of 1μM to measure uptake. It is possible to use a lower concentration of radiolabel and add unlabeled pentamidine up to the desired

concentration. No uptake of pentamidine by HAPT1 will be observed in the LAPT1 experiments, as the permeant concentration is high enough to fully saturate HAPT1. To study HAPT1 or LAPT1 in the *T.brucei* wildtype strain S427, ^{3}H-pentamidine uptake by the P2 transporter must be prevented by saturating it with a final concentration of 1mM adenosine, which can be added to the radiolabel solution. As the number and type of transporters for diminazene uptake were unknown, a series of experiments were conducted using a range of different permeant concentrations (0.02 μM, 0.05 μM and 2 μM).

The assay (see figure 2)

Solutions containing various concentrations of test compounds in assay buffer were added to the radiolabel, where appropriate, and vortexed thoroughly. Assay buffer without inhibitor was added to determine 100% uptake of the permeant. 100μl of the mixture was added to numerically labelled 1.5 ml eppendorf tubes which had the lids removed and contained 250μl of oil mixture (di-n-butylphthalate (BDH) and mineral oil (Sigma) at a 7:1 ratio.) The radiolabel/test compound solution forms an isolated bubble on the top of the oil layer. 100μl of cells were added to each eppendorf and incubated for 60 seconds for pentamidine uptake or 30 seconds for diminazene, mixing the cells to mix with the radiolabel and the test compound. After the incubation period 1ml of ice-cold stop-solution (250 μM of unlabeled pentamidine or diminazene in assay buffer, which will fully saturate the transporter) was added to stop further mediated uptake. Some of the permeant will stick to the outside of the cells instead of being internalised and it is therefore important to eliminate this value from the final count. The control involved adding ice-cold cells and radiolabel simultaneously onto

the oil layer, with a period of zero incubation by adding the stop solution immediately followed by immediate centrifugation. Any apparent uptake observed will not be through mediated uptake and can be deducted from the counts. Centrifugation at 13000 rpm for 1 minute at room temperature was performed to condense the cells into a pellet at the bottom of the oil layer and therefore isolate them from the rest of the radiolabel. The cells contained the radiolabeled permeant accumulated during the assay. The eppendorfs were flash frozen in liquid nitrogen before removing the pellets using tube cutters and transferring them to corresponding numerically labelled scintillation vials. 250 µl of SDS (2%) was added to break open the cells and fully expose the radiolabel present inside. The pellets were left for 20 minutes to allow the SDS to work , after which, 2 mls of scintillation fluid was added to each vial. The amount of accumulated radiation was determined in a liquid scintillation counter. Uptake of each concentration of test compound was performed in triplicate.

1   Potential inhibitor + radiolabel (^{3}H –Pentamidine or ^{3}H– Diminazene).   2   Cells are added and incubated with the inhibitor/radiolabel mixture.

oil laver

3
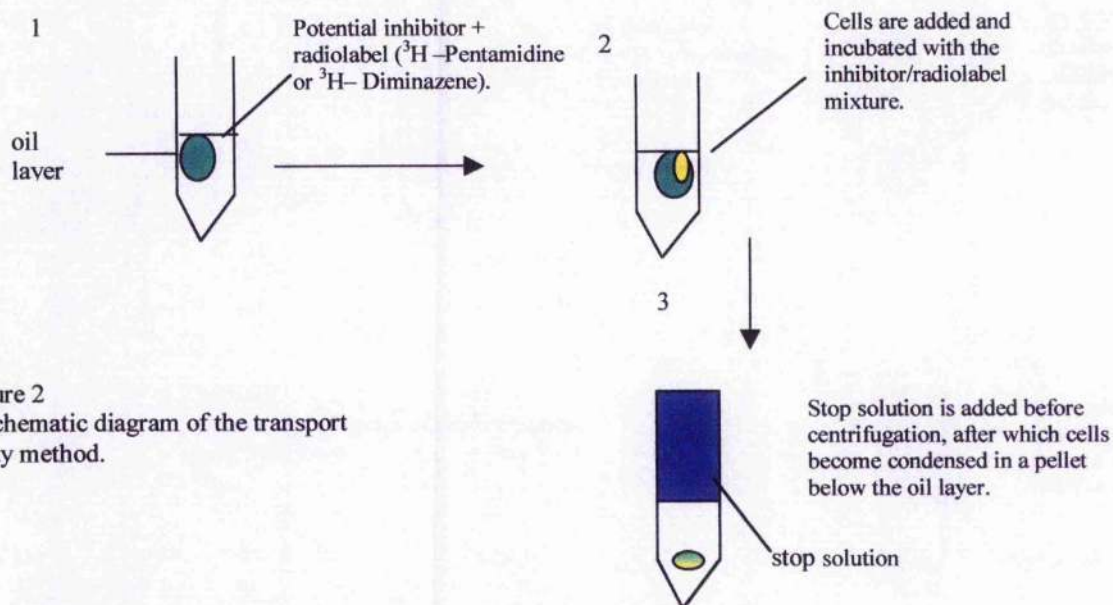
Figure 2
A schematic diagram of the transport assay method.

Stop solution is added before centrifugation, after which cells become condensed in a pellet below the oil layer.

stop solution

Data Analysis

Each experiment was performed in triplicate unless otherwise stated. Full dose-response curves with a minimum of three points over a suitable range, were fitted using either FIG P (Biosoft) or Prism (Graphpad) computer packages and the Michaelis-Menten constant, K_m and maximum transport rate, V_{max} were calculated. The 50% inhibition value, IC_{50} was established from the inhibition profiles of the different test compounds. This value was then used in the Cheng-Prusoff equation, shown below, to determine the inhibition constant K_i.

$$K_i = IC_{50}/[1+(L+K_m]$$

Where L is the permeant concentration.

K_i is a measure of the substrates' ability to bind to the transporter but does not indicate that the ligand is being transported across the surface membrane. The Cheng-Prusoff equation can only be applied in the case of a competitive inhibitor. There are several indicators to suggest that the test compounds were demonstrating competitive inhibition, therefore indicating that the equation is valid. The permeant is always taken up by mediated transport and can be overcome by a high concentration of

substrate. The slope coefficients of the fitted curves are always close to -1, which is associated with monophasic competitive inhibition. In the cases where the possibility of biphasic competition was apparent, an F test, using Prism, was performed to determine statistical significance. All assays were performed in triplicate and are depicted as average and standard errors.

Chapter 3

Inhibition of ^{3}H- Pentamidine uptake by various diamidines and phenantridines

Part 1: Diamidine analogues with furan rings as inhibitors of ^{3}H-Pentamidine uptake by HAPT1 and LAPT1 in *T.brucei brucei* bloodstream forms.

Results

Experiments were carried out using transport assays to determine the ability of three diamidine analogues DB75, DB544 and DB629 to inhibit the uptake of ^{3}H-pentamidine by HAPT1 in *T. brucei* (wild-type strain 427). Diamidine test compounds were mixed with the permeant and incubated with the trypanosomes for 60 seconds before being spun down through an oil layer to form a pellet containing the radiolabeled pentamidine that is accumulated during the assay.

The test compounds are aromatic diamidines like pentamidine, with the pentane 1,5 diether replaced with a furan ring. DB75 is the prototype furan diamidine with the benzamidine groups at positions 1 and 4 of a central furan moiety. DB544 also has methyl groups (CH_3) attached to the furan ring to form a 2,3 dimethylfuran diamidine. In DB629 the methyl groups are on the benzene rings instead, at the ortho position relative to the furan ring. (Chemical structures are shown in Table 1).

All three compounds had some effect on the uptake of ^{3}H-Pentamidine by HAPT1 and the results are presented as inhibition plots (Figure 3.1) which show the inhibitor concentration plotted against ^{3}H-pentamidine uptake (pmol $(10^7$ cells$)^{-1}s^{-1}$). IC$_{50}$ values have been calculated from the inhibition profiles of the test compounds and are shown in the figure legends. Figures 3.1 A and B shows the dose dependent inhibition of mediated pentamidine uptake by DB544 with an IC$_{50}$ value of 22.7 μM and hill slope close to -4. The second test compound DB75 also shows inhibition of permeant uptake with a higher

IC_{50} of 42.2 µM. However, the plot has not decreased to zero at a concentration of 250

µM. This suggests that the uptake of pentamidine is not fully inhibited by DB75 and
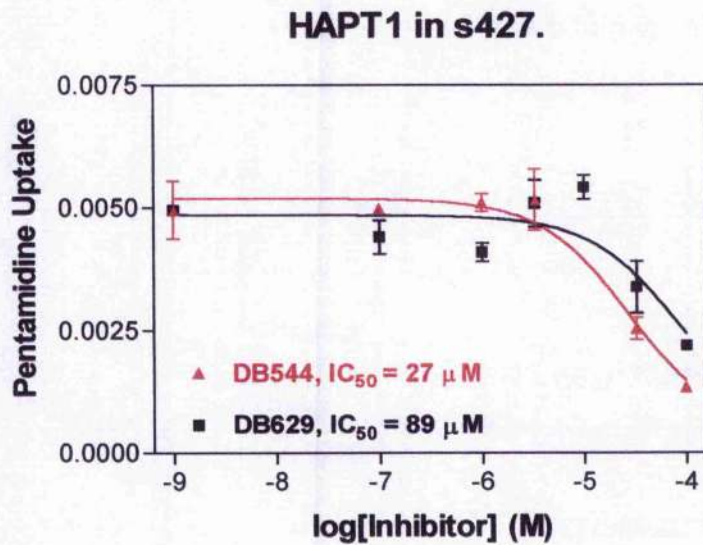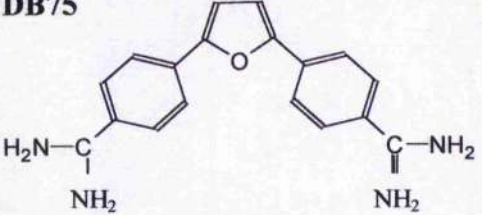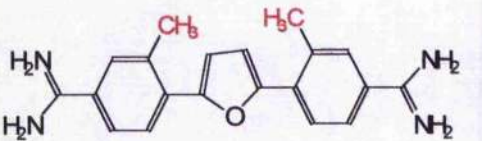
therefore HAPT1 is not fully saturated.

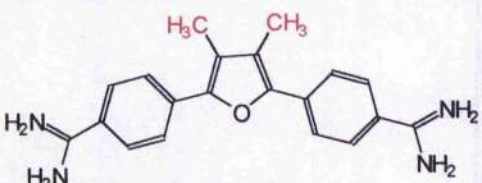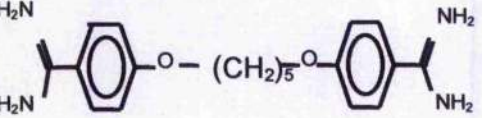**A**
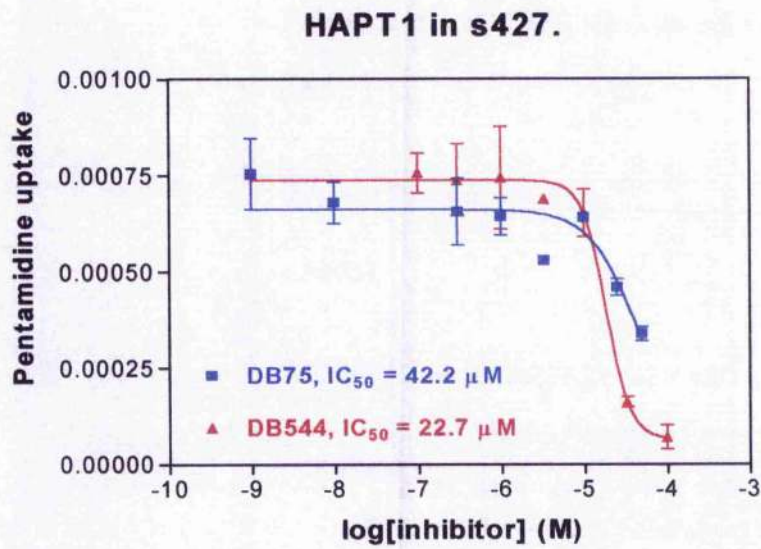
B

FIG 3.1. Inhibition of HAPT1 mediated ^{3}H-Pentamidine uptake by diamidine analogues in *T.brucei brucei* bloodstream forms, strain 427 wild type. Uptake of 10nM ^{3}H-Pentamidine was measured over a 60 second period in the presence or absence of potential competitive inhibitors, which were, unlabeled diamidine analogues with a furan ring and variations between the position of R groups The compounds are as indicated, DB75 (■), DB544 (ᴧ) and DB629 (■). The IC50 values calculated from the inhibition profiles are shown in the graph legends. The results are representative of those obtained from a minimum of 3 repeats of the same experiment. Each point is the average of triplicate determinations and variation is specified in the standard error bars.

Graph B, again shows the inhibition profile of DB544 with an IC50 of 27 μM. DB629 also appears to have some affinity for HAPT1 with an IC50 of 89 μM which is considerably higher than that of DB544 and therefore DB629 has a lower affinity for HAPT1.

K_i values were calculated for each of the three compounds using the Michaelis-menten equation (Table 1). DB75 had low affinity for HAPT1 with a K_i of 1.2 μM ± 20.2 and did not fully inhibit mediated uptake of pentamidine at 250 μM. This is a relatively high concentration of compound considering that HAPT1 is inhibited by extremely small concentrations of pentamidine, with a K_m value of 0.036 μM. DB629 has a slightly higher affinity for HAPT1 with a K_i of 30.9 μM ± 14.2 and also has the addition of methyl groups on both benzene rings. This suggests that the transporter receptor site favours a structure with R groups at this position or may just favour the presence of methyl groups. This seems to be confirmed by the K_i value of DB544 (15.3 μM ± 2.6) which, though, lower, was not significantly different from the one for DB629. However, it was significantly lower than the value for DB75 (P< 0.05). [7] Since DB544 has methyl groups attached to the furan ring it appears that the position of the methyl groups may influence the binding to the transporter. Also, we can suggest that the presence of methyl groups is important in binding to the transporter as DB75, which lacks these structures, has the lowest affinity to HAPT.

Table 1

Ki values, for the diamidine analogues that inhibit the uptake of ^{3}H-Pentamidine permeant by HAPT1, were calculated from the IC_{50} values obtained from transport assay experiments such as those shown in Fig 3.1. Each experiment was carried out between 3-5 times and the K_i values represent average and standard error. The methyl groups located at different positions on the molecule are shown in red. As pentamidine is the original substrate found to be taken up by HAPT1, K_m for pentamidine is also included. All values have been obtained through experiments performed by Dr. H de Koning, Denise Candlish and myself.

Compound name and biochemical structure	Ki (µM)	Number of repeats
DB75	71.2 ± 202	4
DB629	30.9 ± 14.2	3
DB 544	15.2 ± 2.6	5
Pentamidine	0.036 ± 0.006 (K_m)	3

However, if we look at the low K_m value of pentamidine for HAPT1 and compare this to the K_i values of any of the DB compounds it is obvious that none of them have particularly good affinity for HAPT1. Therefore, the carbon chain must play an important role in determining how well a compound is taken up by this transporter because substituting the chain for a furan ring leads to an extreme increase in Ki and therefore a decrease in affinity for HAPT1.

The same experiments were also used to determine whether DB75, DB544 and DB629 inhibited the uptake of ^{3}H-Pentamidine by LAPT1 (Figure 3.2). It was found that DB75 did not inhibit pentamidine uptake and therefore is not taken up by LAPT1. However, both DB544 and DB629 showed dose dependant inhibition of mediated
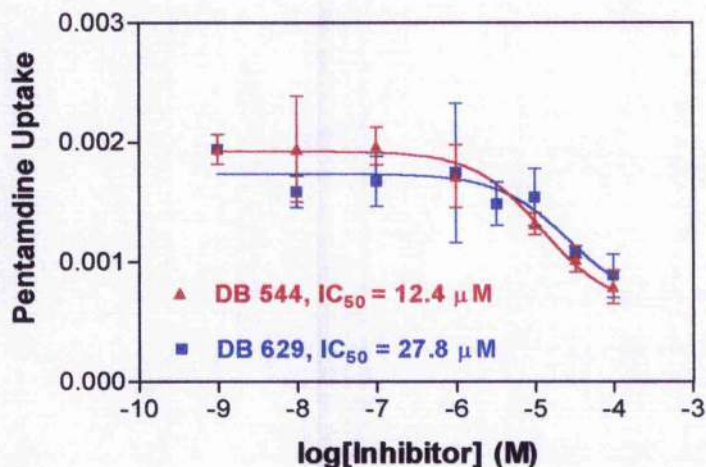
FIG 3.2. **Inhibition of LAPT1 mediated ^{3}H-Pentamidine uptake by DB544 and DB629 in** *T.brucei brucei* **bloodstream forms, strain 427 wild type.** Uptake of 1µM ^{3}H-Pentamidine was measured over a 60 second period in the presence or absence of unlabeled diamidine compounds DB544 (▲) and DB629 (■) which have methyl groups at different positions. Each point is the average of triplicate determinations and variation is specified in the standard error bars.

pentamidine uptake with IC_{50} values of 12.4 μM and 27.8 μM, respectively (n = 1). This suggests that although both diamidine analogues have poor affinity for HAPT1 they have good affinity for LAPT1 when compared to the original substrate pentamidine which has a Km of 56.17 μM. The pattern of order of binding affinity for LAPT1 is the same as for that of HAPT1 with DB75 having little or no affinity for both transporters and DB544 having the highest affinity for both. However, several more experiments using DB544 and DB629 as inhibitors for ^{3}H-Pentamidine uptake by LAPT1 should be conducted to allow Ki values to be calculated and ultimately gain a more accurate overview of the inhibitory profiles of these compounds.

Part 2: Benzamidine compounds, diamidine analogues and phenantridines as potential inhibitors of ^{3}H-Pentamidine uptake in _Trypanosoma brucei brucei_, bloodstream forms.

A set of benzamidine compounds with varying functional groups located at different positions of the ring (figure 3.3) were tested for the inhibition of ^{3}H-pentamidine uptake by both HAPT1 and LAPT1 in _T.brucei brucei_ bloodstream forms. Experiments consisted of transport assays as described in the previous section. The molecule benzamidine consists of a benzene ring attached to an amidine group. This structure forms the end groups of the aromatic diamidine, including pentamidine and DB75. Pentamidine, which has high affinity for both HAPT1 and LAPT1, is constructed of two benzamidine molecules linked by a pentanediether. Using benzamidine as an inhibitor of pentamidine uptake will determine whether or not the presence of a benzamidine moiety is sufficient for the recognition by HAPT1 or LAPT1.

Increasing concentrations of benzamidine, up to 1 mM, were used to inhibit the uptake of ^{3}H-pentamidine by LAPT1 and was found to have no effect on mediated pentamidine uptake. Therefore, an IC_{50} value could not be obtained. In addition 3-aminobenzamidine and 4-aminobenzamidine were also used to test for inhibition of ^{3}H-pentamidine uptake by both HAPT1 and LAPT1. These compounds also had no effect on mediated pentamidine uptake by either transporter, up to a maximum concentration of 10 mM. Finally, compounds 2-hydroxybenzamidine and 4-hydroxybenzamidine, containing hydroxyl groups at the positions indicated, were tested in an identical manner for competitive inhibition of pentamidine uptake by HAPT1 and LAPT1. 2-hydroxybenzamidine had no effect on pentamidine uptake by LAPT1 or HAPT1, up to a

Figure 3.3: Benzamidine compounds
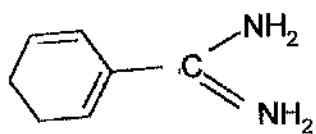
Benzamidine
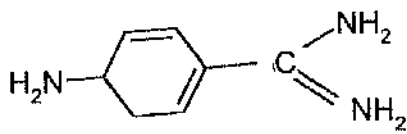
4-aminobenzamidine
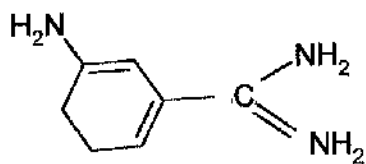
3-aminobenzamidine
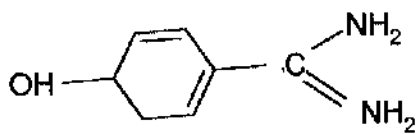
4-hydroxybenzamidine
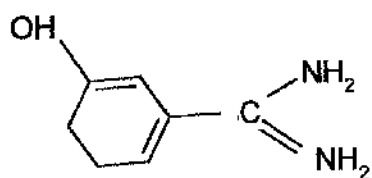
2-hydroxybenzamidine

concentration of 4 mM. The compound was not soluble at higher concentrations. 4-hydroxybenzamidine presented dose dependent inhibition of ^{3}H-pentamidine by HAPT1. An IC_{50} value derived from the inhibition profile was 2.9 ± 1.1 mM (n = 3). This is an extremely high value for HAPT and indicates that the compound has a very low affinity for this transporter. 4-hydroxybenzamidine had no clear effect on LAPT1. It appears that both transporters slightly favour a hydroxyl group at position 4 but overall the benzamidine compounds have no effect on uptake of pentamidine and therefore are not recognised by the transporters.

Various other diamidine compounds were also tested for the inhibition of ^{3}H-Pentamidine by both HAPT1 and LAPT1. The first to be tested was DAPI (4',6-diamidino-2-phenylindole) that is commonly used as a blue fluorescent DNA probe due to its DNA intercalating properties. The structure has a diamidine composition where the carbon chain has been replaced with a pyrrole ring fused to one of the benzene rings, forming indole. (Table 2). DAPI was found to dose-dependently inhibit the uptake of ^{3}H-Pentamidine by both HAPT1 and LAPT1. (Figure 3.4 A and B) DAPI appeared to have a relatively low affinity for HAPT1 at 26.6 ± 5.8 μM when compared to the original substrate pentamidine which has a K_m of 0.036 μM for the same transporter. However, surprisingly, DAPI has an extremely high affinity for LAPT1 with a K_i of 13.6 ± 13.6 μM compared with pentamidine (K_m of 56.2 ± 8.2 μM, P< 0.05) LAPT1, therefore, appears to favour the structure of DAPI over that of pentamidine, whereas HAPT1 does not.
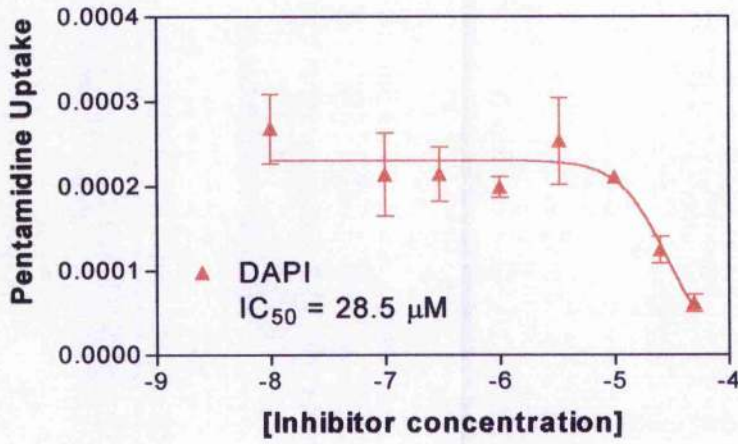
B
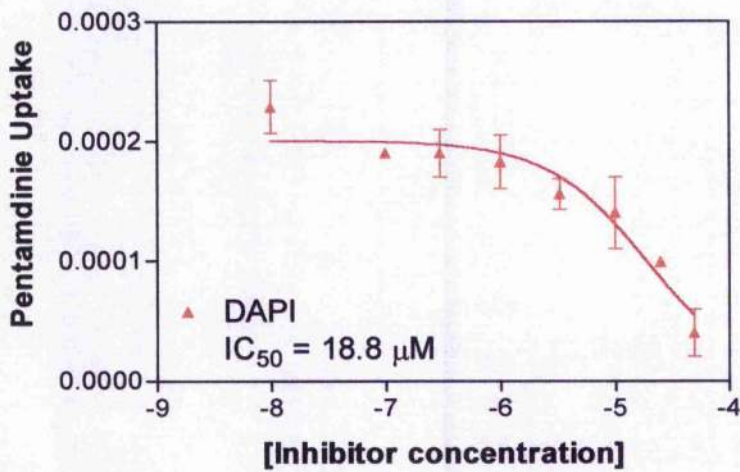
Figure 3.4 A and B: The inhibition of ^{3}H-pentamidine uptake by DAPI in HAPT1 (A) and LAPT1 (B). IC_{50} vlaues were calculated from the inhibition profiles and are 28.5 μM and 18.8 μM for HAPT1 and LAPT1, respectively (n = 1).
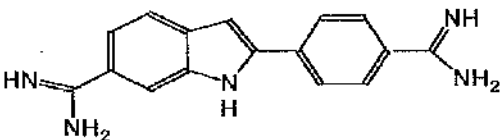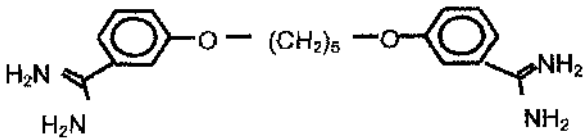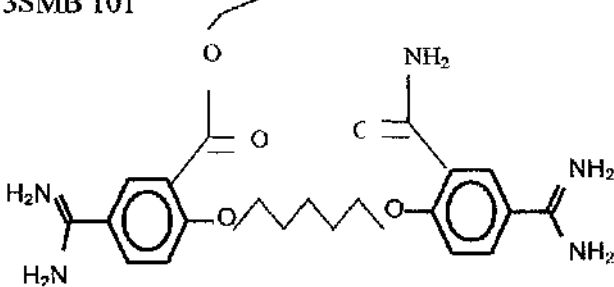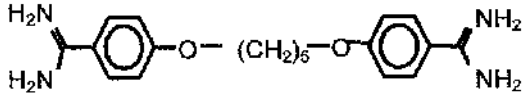
The second diamidine compound to be tested for the inhibition of ^{3}H-Pentamidine uptake was meta-pentamidine. This compound has the amidine groups not at the para-position as seen in pentamidine, but at the meta-position, (Structure see Table 2) Meta-pentamidine was used to inhibit the uptake of ^{3}H-pentamidine by HAPT1 and demonstrated dose dependent inhibition of the transporter, with a K_i value of 65.1 ± 18.1 μM. It is clear that the change of amidine groups from the para to the meta position greatly reduces the affinity of a compound for HAPT1.

Finally, a new test compound synthesised for activity against trypanosomes, labelled 3SMB 101 was used to inhibit mediated uptake of ^{3}H-Pentamidine by HAPT1. (Table 2). This compound uses the pentamidine structure as a scaffold with an amido and an ethoxy group added to position 2 of either benzene ring (2-ethoxy,2 amido-pentamidine). 3SMB 101 was found to inhibit ^{3}H-pentamidine uptake with a K_i of 109 ± 17.45 μM, which is relatively low. It therefore appears that HAPT1 favours a simpler structure, but it is impossible to say whether the size or the charge of the substitutions (or both) is responsible for the loss of affinity.

The affinity of the phenantridine ethidium bromide (homidium) for HAPT1 and LAPT1 was also investigated. This compound was chosen for two reasons. The first is that it is a widely used trypanocide for the treatment of bovine trypanosomiasis, thought to enter the trypanosome by diffusion. As uptake is not thought to be mediated by a surface

Table 2

K_i values, for the diamidine compounds that inhibit the uptake of ^{3}H-Pentamidine permeant, were calculated from the IC_{50} values obtained from transport assay experiments. Values are presented for both the low and high affinity transporters. Each experiment was carried out between 2-4 times and the values

Diamidine Structure and name	LAPT	HAPT	Number of Repeats	
	K_i (μM)	K_i (μM)	LAPT	HAPT
DAPI 	13.6 ± 3.4	26.6 ± 5.8	2	3
Meta-pentamidine 	ND	65.1 ± 18.1	ND	4
3SMB 101 	ND	109 ± 17	ND	2
[a]Pentamidine 	56.17 ± 8.29	0.036 ± 0.06	4	3

shown represent averages and standard errors.

ND, not determined

[a] K_m values are shown for pentamidine rather than K_i values.
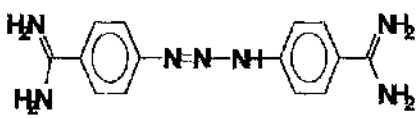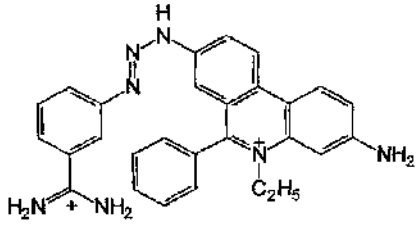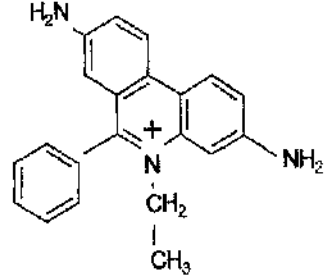
membrane transporter it would be interesting to find out if Ethidium bromide has any affinity for HAPT1 or LAPT1. The second reason is that Berenil and Isometamidium (ISMM) have been previously tested for the inhibition of ^{3}H-pentamidine uptake by both pentamidine transporters. ISMM is comprised of both Berenil and ethidium bromide molecules and therefore an insight into the recognition motifs of HAPT1 and LAPT1 can be gained by comparing the K_i values of the three compounds. Ethidium bromide consistently showed dose dependent inhibition of ^{3}H-pentamidine uptake by HAPT1, but with a high K_i value of 99.2 µM ± 17.9 µM indicating a low affinity for this transporter. If these results are compared with those found for Berenil and ISMM (Table 3). Berenil shows a slightly lower K_i value of 63.1 µM but ISMM shows a significantly lower K_i value of 3.60 µM. It therefore appears that out of the three compounds, ISMM has the highest affinity for HAPT1, followed by Berenil and finally by ethidium. ISMM, a combination of the other two compounds, is more easily recognised by HAPT1 than either ethidium bromide or Berenil on their own.

The same experiments were carried out for LAPT1, where ethidium bromide inhibited the uptake of ^{3}H-pentamidine, with the surprisingly low K_i of 18.83 ± 7.34 µM. Ethidium bromide has a high affinity for LAPT1. Of this class of compounds Berenil displayed the lowest affinity ($K_i = 494.2$µM) and ISMM again showed the highest affinity ($K_i = 7.21$µM) (Table 3). It appears that LAPT1 favours the phenanthridine structure over Berenil but also that when both compounds are combined to form ISMM; the affinity for LAPT1 is significantly increased (P < 0.05). Comparing the results for HAPT1 and LAPT1 suggests that theses transporters may have slightly different recognition motifs. In an attempt to discover the biological function of these transporters the compounds

Choline chloride (10 mM), Lactic acid (10 mM), Palmitic acid and Lauric acid (both at 1.3 mM), products found in the host environment, were tested for their ability to inhibit the uptake of ^{3}H-pentamidine by both HAPT1 and LAPT1. None of the compounds had any effect at the tested concentrations.

Table 3

K_i values for the inhibition of ^{3}H-pentamidine uptake by common bovine trypanocides are shown below for both HAPT1 and LAPT1. Each experiment was repeated three times and the K_i value is therefore an average value of three individual experiments. Dr H. de Koning and Lynsey Wallace carried out the work on Berenil and isometamidium.

Name and Structure of Compound	HAPT	LAPT
	Ki (μM)	Ki (μM)
Berenil	63.09	494.2
Isometamidium	3.60	7.21
Ethidium Bromide	99.18	18.83

Part 3: Diamidine compounds of varying carbohydrate chain length as inhibitors of ^{3}H-Pentamidine uptake in *Trypanosoma brucei brucei* bloodstream forms.

Results

Transport assays performed exactly as described in the previous section, were carried out using diamidine compounds with increasing carbohydrate chainlength to inhibit the uptake of ^{3}H-Pentamidine ($1\mu M$) by LAPT1 in *T. brucei brucei* bloodstream forms. All of the diamidines tested, which are listed with diagrams of their chemical structures in Table 4, consistently and dose dependently inhibited mediated pentamidine transport by LAPT1. However, the extent at which they individually compete with pentamidine for the same transporter varies dramatically and appears to be dependent on the central carbon chain.

The results were evaluated in graphs such as those demonstrated in Figure 3.5 which show the inhibitor concentration plotted against LAPT1-mediated ^{3}H-Pentamidine uptake $(pmol(10^7 \text{ cells})^{-1} s^{-1})$. The IC_{50} values were calculated from the inhibition profiles of the diamidines and are representative of values obtained for several repeats of the same experiment. Figure 3.5 A shows the effect of pentamidine and hexamidine on transport of the permeant. As pentamidine is the original substrate found to be transported by LAPT1 it is often useful to use it as a control against which the affinity of other test compounds can be compared. Hexamidine appears to have a higher affinity for LAPT1 than pentamidine with IC_{50} values of $49 \mu M$ and $161 \mu M$, respectively. Both compounds are identical apart from the number of carbon molecules in the connecting chain (Table 4 and Figure 3.7 C). The addition of another carbon molecule to hexamidine to produce
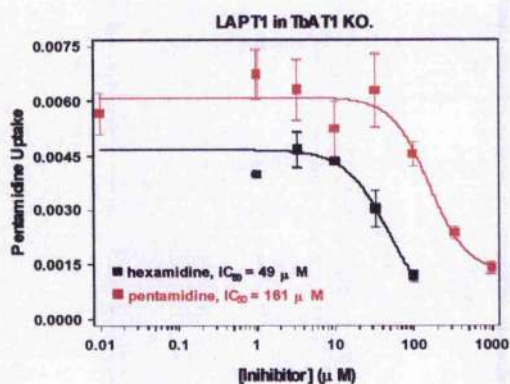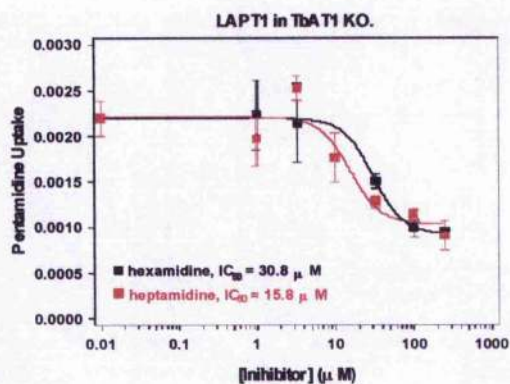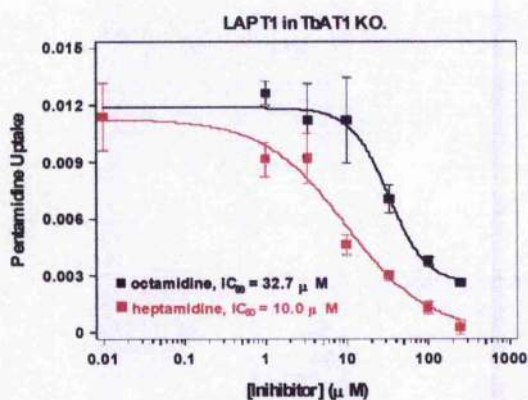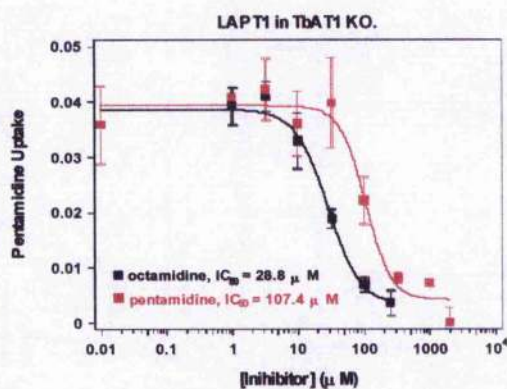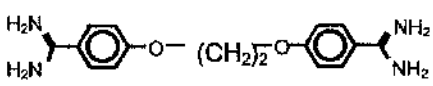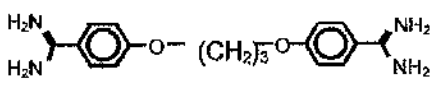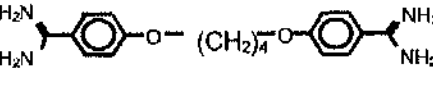
FIG 3.5. **Inhibition of LAPT1 mediated ^{3}H-Pentamidine uptake by diamidine analogues in *T.brucei brucei* bloodstream forms, strain ΔTbAT1.** Uptake of 1μM ^{3}H-Pentamidine was measured over a 60 second period in the presence or absence of potential competitive inhibitors, which were, unlabeled diamidine analogues of varying carbohydrate chainlength, as indicated. A, Pentamidine (■) and Hexamidine (■), B, Hexamidine (■) and Heptamidine (■), C, Heptamidine (■) and Octamidine (■), D, Octamidine (■) and Pentamidine (■). The IC50 values calculated from the inhibition profiles are shown in the graph legends. The results are representative of those obtained from three repeats of the same experiment. Each point is the average of triplicate determinations and variation is specified in the standard error bars.

heptamidine leads to a further increase in the affinity for (Figure 3.5 B) In this case hexamidine displays an IC_{50} value of 30.8 μM, while the value for heptamidine was 5.8 μM, denoting a higher affinity for the transporter. Figures 3.5 C and D show the effect of octamidine, possessing 8 methylene groups on pentamidine uptake alongside inhibition profiles of heptamidine and pentamidine, respectively. Surprisingly, octamidine appears to display a lower affinity for HAPT1 than heptamidine, with K_i values of 21 ± 7 μM and 14 ± 2 μM, respectively, but this difference was not statistically significant (P>0.05). All hillslopes for the sigmoid plots presented are close to -1 consistent with a single transporter model showing inhibition of mediated drug uptake.

Several other diamidine compounds of shorter chain lengths were also used as potential inhibitors, in previous experiments by Dr. H. de Koning, L.Wallace and D.Candlish. The K_i values for diamidines with 2-8 carbons in the central chain are presented in Table 4. Propamidine displayed the highest K_i value of 316 μM for LAPT1. The K_i value gradually decreased as the number of methylene groups increased. Butamidine has the lower K_i of 196.83 μM. Hexamidine, heptamidine and octamidine all display relatively high affinity for HAPT1, and their K_i values are not statistically different.

Table 4

K_i values, for the diamidine compounds that inhibit the uptake of ^{3}H-Pentamidine permeant, were calculated from the IC_{50} values obtained from transport assay experiments such as those shown in Fig 3.5. Values are presented for both the low and high affinity pentamidine transporters. Each experiment was carried out between 3-5 times and the values shown represent averages and standard errors.

Diamidine Structure and name	LAPT1	HAPT1	Number of repeats	
	K_i (μM)	K_i (μM)	LAPT1	HAPT1
Ethamidine H_2N–C(NH$_2$)–C$_6$H$_4$–O–(CH$_2$)$_2$–O–C$_6$H$_4$–C(NH$_2$)NH$_2$	ND	> 100	0	4
Propamidine H_2N–C(NH$_2$)–C$_6$H$_4$–O–(CH$_2$)$_3$–O–C$_6$H$_4$–C(NH$_2$)NH$_2$	315.87 ± 3.13	6.52 ± 1.66	5	5
Butamidine H_2N–C(NH$_2$)–C$_6$H$_4$–O–(CH$_2$)$_4$–O–C$_6$H$_4$–C(NH$_2$)NH$_2$	196 ± 31	3.9 ± 1.4	3	4
[a]Pentamidine H_2N–C(NH$_2$)–C$_6$H$_4$–O–(CH$_2$)$_5$–O–C$_6$H$_4$–C(NH$_2$)NH$_2$	56.17 ± 8.29	0.036 ± 0.006*	4	3
Hexamidine H_2N–C(NH$_2$)–C$_6$H$_4$–O–(CH$_2$)$_6$–O–C$_6$H$_4$–C(NH$_2$)NH$_2$	35 ± 9	0.059 ± 0.010*	4	3
Heptamidine H_2N–C(NH$_2$)–C$_6$H$_4$–O–(CH$_2$)$_7$–O–C$_6$H$_4$–C(NH$_2$)NH$_2$	14 ± 2	0.12 ± 0.01*	3	4
Octamidine H_2N–C(NH$_2$)–C$_6$H$_4$–O–(CH$_2$)$_8$–O–C$_6$H$_4$–C(NH$_2$)NH$_2$	21 ± 7	2.20 ± 0.48*	4	3

ND, not determined.

* Values taken from experiments conducted by Dr.H. de Koning and D. Candlish

[a] K_m values are shown for Pentamidine rather than Ki values.

The difference in binding affinities between propamidine and heptamidine for LAPT1 is large. However, these values are not a measure of drug uptake by LAPT1 in *T.brucei*. We can only measure true drug uptake using a substrate that is radiolabeled, in this case pentamidine. Therefore the K_i is an inhibition constant defines the inhibitor concentration inhibiting 50% of the transporters. In the case of pentamidine the K_m value (56.17 µM) is given because a direct measurement of pentamidine uptake could be taken.

These results suggest that a longer carbon chain length increases binding affinity to LAPT1 and suggest that a chainlength of 7 methylene groups may be optimal. Experiments using the same method and the same range of diamidine compounds, including ethamidine (carbon chain of 2), were carried out to investigate the effects of carbon chain length on the uptake of ^{3}H-Pentamidine (15 nM) by HAPT1. Figure 3.6 shows the inhibition of ^{3}H-Pentamidine uptake (pmol $(10^7$ cells) $^{-1}$ s^{-1}) by butamidine. Unlabeled pentamidine was used as an internal control. It is obvious from this graph that butamidine dose dependently inhibits the uptake of pentamidine. The Hill slope for butamidine is close to -1, indicating that only HAPT1 is being inhibited. This is confirmed by the observation that butamidine only inhibits to the same level as pentamidine in its first (high affinity) phase.
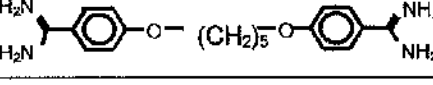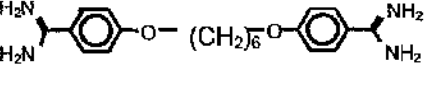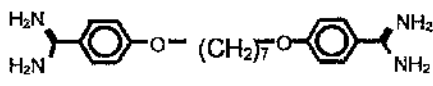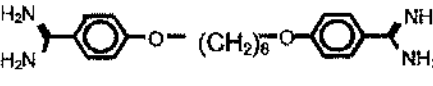
HAPT1-mediated pentamidine uptake in ΔTbAT1.
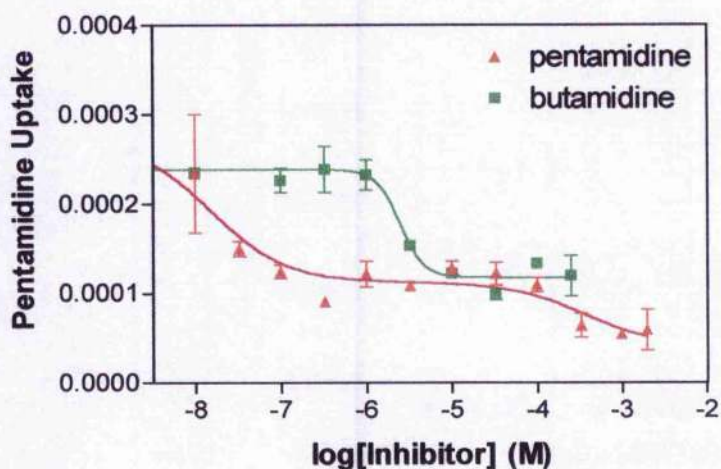
FIG 3.6. **Inhibition of HAPT1 mediated ^{3}H-Pentamidine uptake by Butamidine in *T.brucei brucei* bloodstream forms, strain ΔTbAT1.** Uptake of 15 nM ^{3}H-Pentamidine was measured over a 60 second period in the presence or absence of unlabeled diamidine compound butamidine (■). Unlabeled pentamidine (▲) was used as a control. This experiment was repeated three times and the above graph is representative of the overall result obtained. Each point is the average of triplicate determinations and variation is specified in the standard error bars.

The shift for butamidine to the right of pentamidine shows that butamidine has a higher IC$_{50}$ value and therefore a lower affinity than pentamidine for HAPT1. K$_i$ values of diamidines of increasing carbon chain length for HAPT1 are also shown in Table 4. Ethamidine with the lowest carbon chain length has the highest Ki value of over 100 μM. There is a significant decrease in Ki for propamidine and butamidine (6.35 μM and 3.91 μM, respectively). The Km value for pentamidine is given at 0.036 μM ± 0.006, which is extremely low and shows that pentamidine has the highest affinity for HAPT1. As the

carbon chain length further increases for the compounds hexamidine, heptamidine and octamidine the Ki values also increase from 0.059 μM to 0.125 μM ± 0.001, to 2.20μM, respectively, though the difference between pentamidine and hexamidine is not significant. This data concludes that an aromatic diamidine optimally bind HAPT1 with a carbon chain length of 5. Generally the compounds with the higher number of carbon molecules show a higher affinity for HAPT1. A compound with a 9-methylene chain was not available bit it would be expected to show lower affinity for HAPT1 than octamidine. A summary of the effect of carbon chain length on ^{3}H-Pentamidine uptake by both HAPT1 and LAPT1 is shown in Figure 3.7 A and B. Although the K_i values are of very different concentrations for HAPT1 and LAPT1 due to the kinetics of the individual transporter, a similar pattern can be seen between the two which is clearly presented in the graphs. For both, propamidine and butamidine, with carbon chains of 3 and 4, have very large K_i values and the difference in K_i value between butamidine and pentamidine is extensive. The optimum chain length for HAPT1 and LAPT1 appear to be different being pentamidine and heptamidine respectively. However, all the compounds in this range have fairly high affinities for both transporters. There is an increase in Ki for octamidine, which is associated with both the transporters, this increase being much more pronounced for HAPT1. Statistical analysis reveals that the HAPT1 optimum is 5-6 methylene groups, compared to 6-8 for LAPT1.
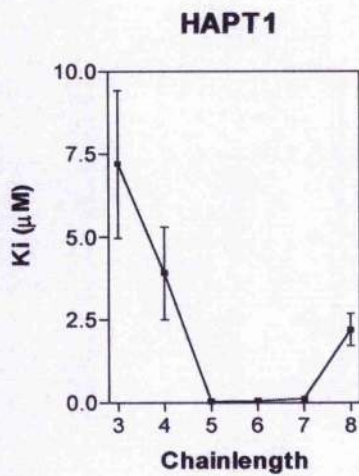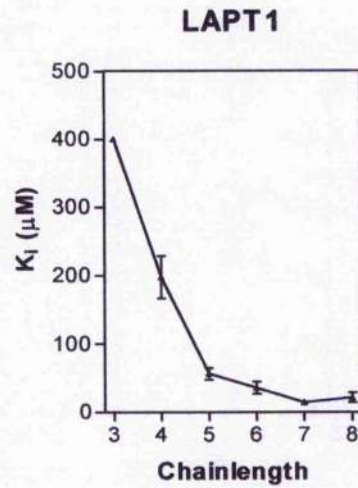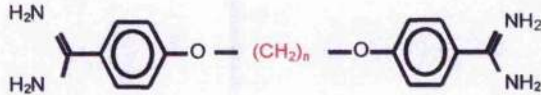
FIG 3.7. **Ki values calculated for the inhibition of HAPT1 or LAPT1 mediated transport of** [3]**H-Pentamidine, by diamidine analogues of increasing carbohydrate chain length.** Uptake of ^{3}H-Pentamidine was inhibited by unlabeled diamidine compounds of increasing carbohydrate chainlength from ethamidine ($2 \times CH_2$) to octamidine ($8 \times CH_2$). The Ki values were calculated from the Michaelis-Menten equation. These values were plotted against the carbohydrate chainlength number for A, the HAPT1 and B, the LAPT1. C, shows the standard diamidine molecular structure with the variable segment indicated in red.

Chapter 4

The uptake of Berenil (Diminazene aceturate) by *Trypanosoma brucei,* bloodstream forms.

A time course experiment, using the transport assay method, as previously described in the Materials and Methods section, was carried out to measure the uptake of ^{3}H-diminazene by bloodstream forms of *Trypanosoma brucei* (wild type). Cells were incubated over increasing time points with fixed concentrations of ^{3}H-diminazene at 0.05 μM or 1 mM. As diminazene is a substrate for the P2 transporter, at 1mM the transporter should be fully saturated and the presence of any other diminazene transporters will become apparent. The same experiment was also carried out using TbAT1 cells that no longer have a functional P2 transporter. Any uptake occurring in these cells will not be through the P2 transporter and will again allow the detection of any other specific diminazene transporters. The results were plotted as diminazene uptake (pmol/10^{7}cells/s) against time (s) (Figure 4A). In the WT cells at 0.05 μM uptake was rapid and linear over a period of 120 seconds with a rate of 8.6 ± 0.5 fmol $(10^7 \text{ cells})^{-1} \text{ s}^{-1}$. However, at 1 mM there was no evidence of mediated uptake. A very small amount of substrate appeared to be taken up, possibly, by passive diffusion into the cells, however this did not reach statistically significant levels (P = 0.8). There was no mediated or unmediated uptake observed in the TbAT1 cells. The uptake of diminazene by *Trypanosoma brucei* is therefore mediated by a single transporter that is likely to be the P2 transporter.

Using the transport assay, unlabelled diminazene was used to inhibit the uptake of ^{3}H-diminazene over a period of 30 seconds (Figure 4B). The reduced incubation time was based upon the rate of uptake of ^{3}H-diminazene observed in the previous timecourse experiment, which appeared to be higher than the uptake of pentamidine by *T.brucei*. ^{3}H-diminazene uptake was inhibited dose dependently by unlabelled diminazene and the inhibition profiles obtained from a repeat of 4 experiments confirmed the presence of a

single, saturable transporter involved in the mediated uptake. The K_m and V_{max} values were calculated and are found to be $0.445 \pm 0.106\mu M$ and 0.049 ± 0.010 pmol/10^7cells/s, respectively (n = 4)

^{3}H diminazene uptake in *T.brucei* bloodstream forms
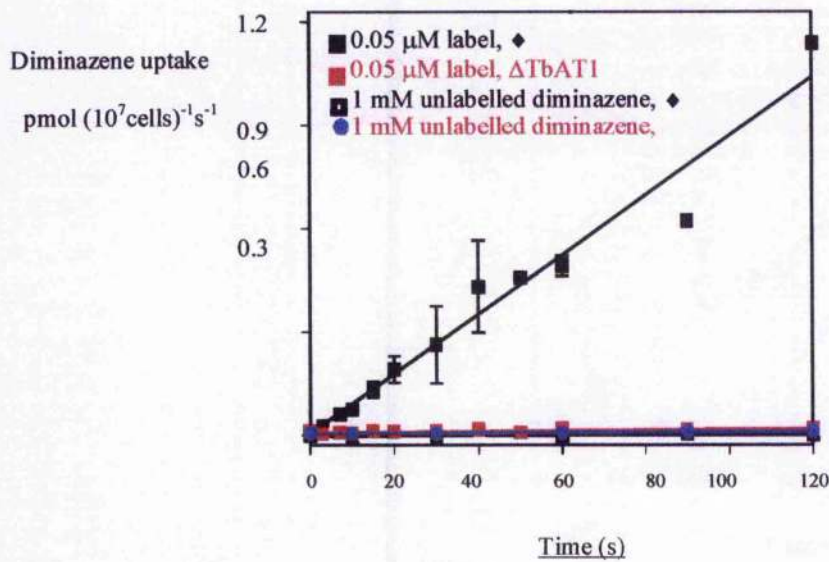
Figure 4A : **Timecourse of ^{3}H-diminazene uptake in *T.brucei* s427(♦) and ΔTbAT1 bloodstream forms.** (A) Uptake of ^{3}H-diminazene, at concentrations of 0.05 μM in the presence or absence of 1mM unlabelled diminazene was measured over increasing time periods in both wild type and TbAT1 knockout bloodstream forms of *T.brucei* (indicated in the figure legend)

Michaelis-Menten high affinity
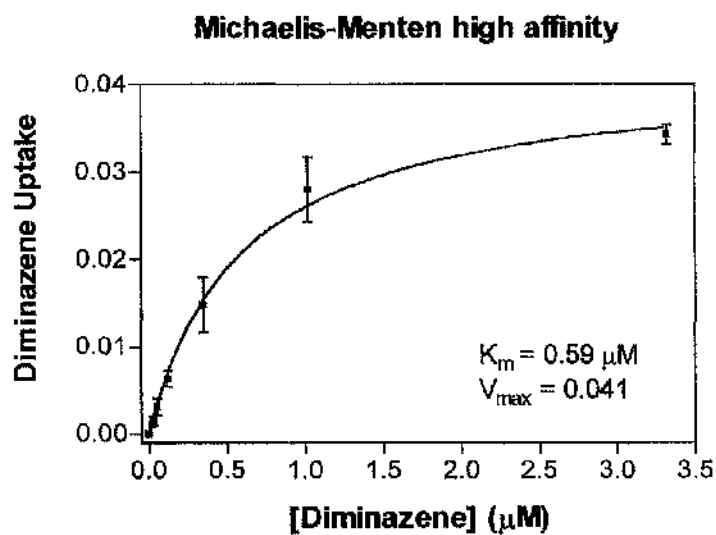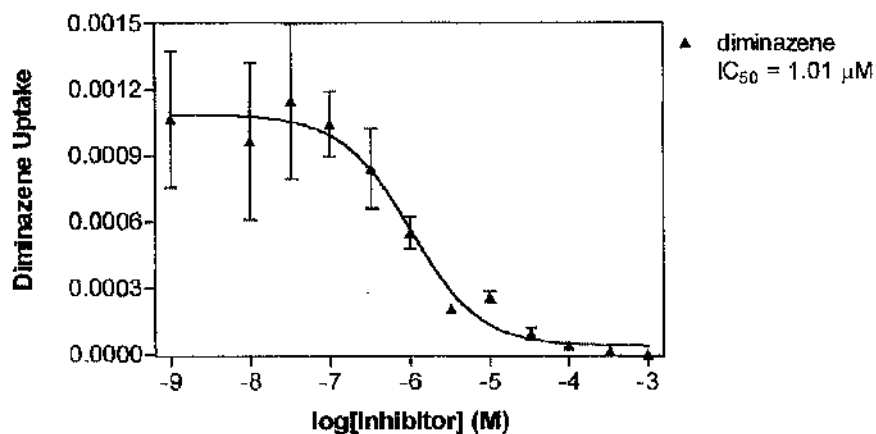
Figure 4B: The inhibition of ^{3}**H-diminazene uptake by unlabelled diminazene in** *T.brucei* **s427.** The uptake of ^{3}H-diminazene at 0.05 μM, was measured over a 30 second period in the presence of unlabelled diminazene. The IC_{50} values were calculated from the inhibition profiles shown in the graph. A Michaelis-Menten plot was used to calculate the K_m and V_{max} values.

Other known substrates of the P2 transporter, adenosine and pentamidine, were used to inhibit the uptake of ^{3}H-diminazene to determine whether P2 is the transporter responsible for transport of the substrate across the membrane. (Figure 4C) Both test compounds inhibited the uptake of ^{3}H-diminazene. After several repeats the Ki values were calculated from the inhibition profiles and pentamidine was found to competitively inhibit ^{3}H-diminazene with a higher affinity ($K_i = 0.21 \pm 0.018\mu M$ (n = 2)) than adenosine ($K_i = 0.25 \pm 0.081$ μM (n = 4)).

Uptake of ^{3}H-diminazene by
***T.brucei* wild type**
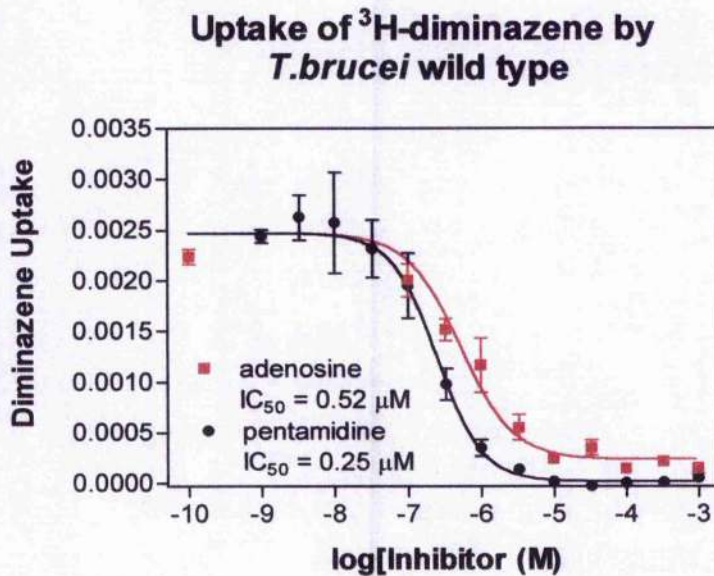
Figure 4C: The inhibition of ^{3}H-diminazene by adenosine and pentamidine. The uptake of ^{3}H-diminazene at 0.05 μM, was measured over a 30 second period in the presence or absence of unlabelled P2 substrates, adenosine (■) and pentamidine (●). The IC_{50} values were calculated from the inhibition profiles shown in the graph.

These results clearly demonstrate that ^{3}H-diminazene is taken up by the P2 transporter. To investigate whether there was any additional contribution from the related P1 transporter, inosine, a substrate for the P1 transporter, was also tested for inhibition of ^{3}H-diminazene. It was observed that inosine had no clear effect on the uptake of ^{3}H-diminazene, and the drug is therefore not taken up by the P1 transporter. (Figure 4D)
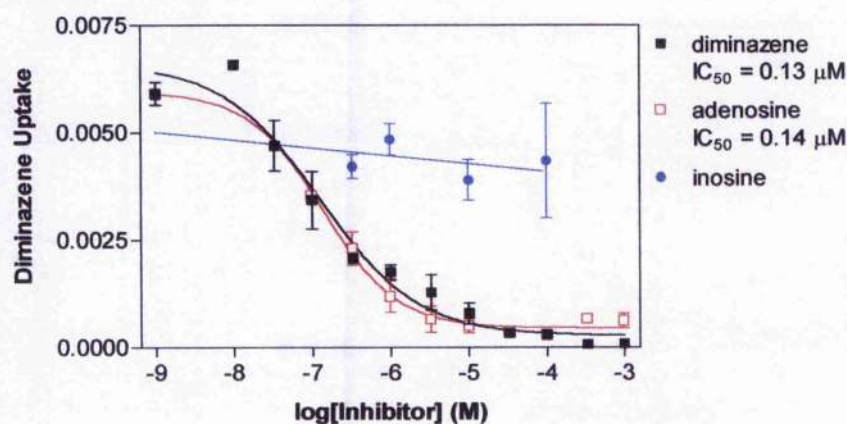
Figure 4D: The inhibition of ^{3}H-diminazene uptake by adenosine, pentamidine and inosine. The effects of inosine, a P1 substrate, on the uptake of ^{3}H-diminazene were measured over a 30 second period. Adenosine and pentamidine, known to inhibit ^{3}H-diminazene uptake from the previous experiment, were used as controls.

We can conclude that the uptake of diminazene is mediated by a single transporter that is identical to the P2 transporter in that it has all the substrate specificities and the saturation profile of this transport system. When this transporter is knocked out there is no uptake and therefore the P2 transporter is the only transporter involved in diminazene uptake.

Chapter 5

Pentamidine uptake and drug

sensitivity in *Crithidia fasciculata*

Part 1: Pentamidine uptake in *Crithidia fasciculata*

[3]H- Pentamidine uptake by *Crithidia fasciculata* (strain HS-6) was measured using the transport assay method described in the Materials and Methods section. However, in this case, the concentration of permeant remained constant at concentrations 1µM or 1mM whilst the time of incubation was varied. The time course is plotted as the rate of uptake of [3]H- pentamidine (fmol $(10^7$ cells)$^{-1}$ s^{-1}) against time. (Figure 5A). At 1µM uptake of [3]H- pentamidine is linear over a period of 2 minutes at a rate of 0.2 fmol $(10^7$ cells)$^{-1}$ s^{-1} as determined by linear regression ($r^2 = 0.998$). This is conclusive evidence of the presence of a pentamidine transporter. However, at 1mM, there is no uptake of permeant, indicating that the pentamidine transporter is saturable and uptake of label is completely inhibited by unlabelled pentamidine at this concentration.

Increasing concentrations of unlabeled pentamidine were then used to inhibit uptake of [3] H- Pentamidine to test for the number of transporters involved. Figure 5B is representative of results obtained from four repeats of the same experiment. In this experiment, pentamidine inhibited the uptake of permeant with an IC_{50} of 0.38µM. Conversion of the same data to a Michaelis-Menten plot yielded the K_m and V_{max} values. The data shown in Figures 5B and C are consistent with a model transporter that is saturable and conforms to Michaelis- Menten kinetics. The average K_m and V_{max} values for pentamidine uptake by *Crithidia fasciculata* are 0.66 ± 0.18µM and 0.054pmol/10^7 cells/s (± 0.033), respectively.

Uptake of Pentamidine by
Crithidia fasciculata
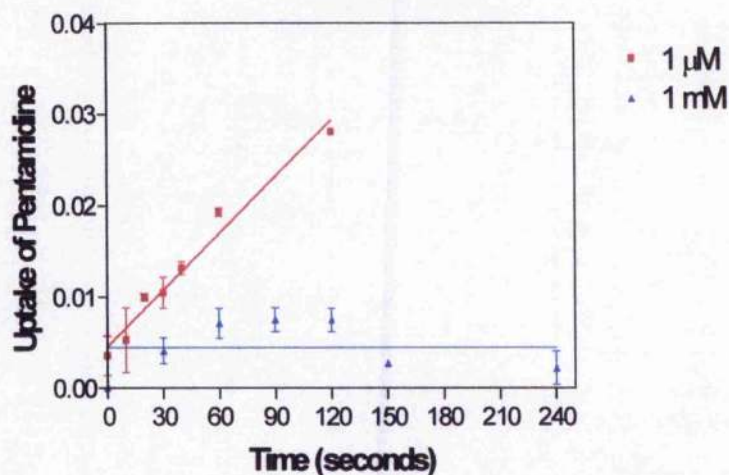
Uptake of [3]H- Pentamidine by *Crithidia fasciculata* (strain HS-6). Cells were incubated with fixed concentrations of permeant at $1\mu M$ or $1mM$ for increasing time periods to measure the uptake of pentamidine (pmols $(10^7 \text{ cells})^{-1} \text{ S}^{-1}$). and thus determine the presence or absence of a specific pentamidine transporter.

Propamidine was also assayed for the inhibition of pentamidine uptake (Figure 5B) and it was found to have a much lower affinity for the transporter than pentamidine, with a Ki of $128 \pm 38\mu M$.

Pentamidine Uptake in *Crithidia fasciculata*

Michaelis-Menten plot
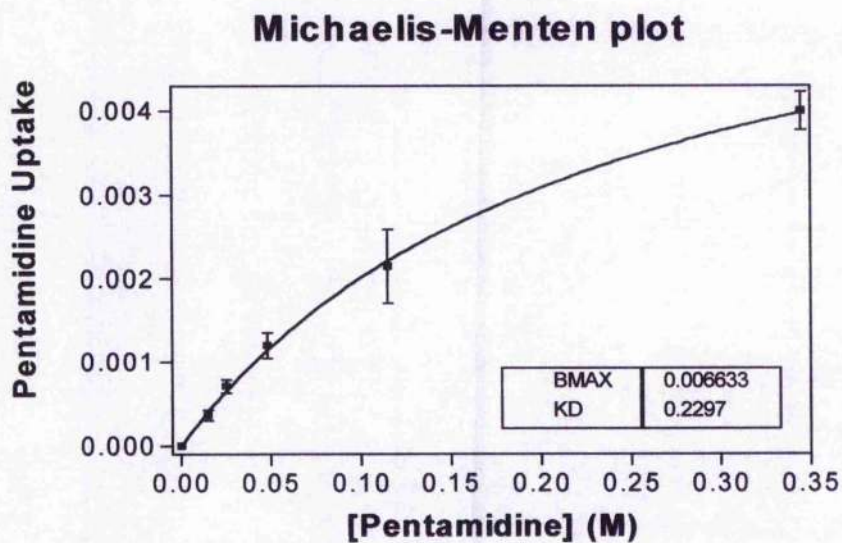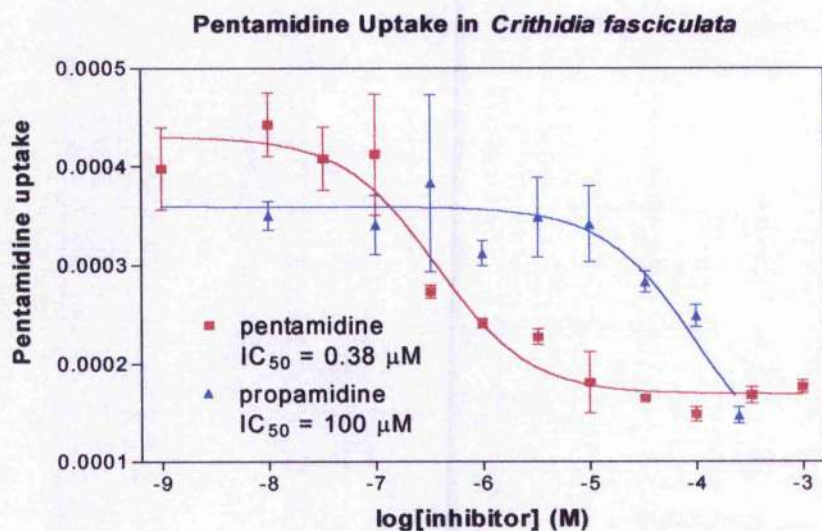
The Inhibition of [3] H- Pentamidine in *C.fasciculata* by pentamidine and propamidine. Figure 5B shows the dose dependant inhibition of [3] H- Pentamidine by diamidine compounds pentamidine (■) and propamidine (▲). The IC_{50} values were calculated from the inhibition profiles. Figure 5C is a Michaelis-menten plot showing the rate of uptake of 1μM [3]H- pentamidine over 60 seconds by *C.fasciculata*.

Finally, pentamidine analogues butamidine and diminazene were also tested for their ability to inhibit pentamidine uptake by competing for the identified transporter. A time course for the uptake of ^{3}H-diminazene by *C.fasciculata* showed that there was no evidence of mediated uptake of diminazene, by this organism (data not shown). However, when diminazene was used as an unlabelled inhibitor of ^{3}H-pentamidine, dose dependent inhibition was observed with a Ki value of 8.02 μM (S.E. 4.43 μM). Butamidine appeared to have a good affinity for the pentamidine transporter with a low Ki value of 0.49 μM (SE. 0.017 μM). Both these experiments were only performed twice and further repeats should be carried out to obtain more conclusive results.

A Modified Alamar Blue Assay as a method to determine drug sensitivity of *Crithidia fasciculata.*

A method was required to determine the sensitivity of *Crithidia fasciculata* (strain 11S-6) to pentamidine and various other trypanocides. This is important to form a correlation between the drug sensitivity and drug uptake by the parasite.

There are several methods available for drug sensitivity testing in kinetoplastids but the Alamar Blue assay appeared to be the most reliable and easily reproducible of the various options. Alamar Blue was originally used to examine the cytotoxic effects of compounds on mammalian cells. It has since been found to be successful in the determination of drug sensitivities in human infective African trypanosomes. (Raz et al, 1997) The dye employed in the assay has fluorescent properties and is metabolised by the parasites and therefore can be used to indicate the extent of metabolic activity. As the metabolic activity will be highest during cellular proliferation in logarithmic growth phase, there is a linear relationship between the fluorescent signal emitted and the number of dividing cells. It was assumed that it would be possible to use Alamar Blue to study drug sensitivity in *C.fasciculata* based on the similarities in biochemical function between trypanosomes and *Crithidia.*

Stocks of the test compounds were prepared at 500 μM and 200 μls of each were added to the first column of a 96 well plate, with a separate row for each drug, in duplicate. The remaining wells contained 100 μls of LIT medium and doubling dilutions were made by

transferring 100 μls of stock drug into the adjacent well to mix with the medium. This was repeated along the row using a multi-channel micropipette. DMSO, which rapidly kills *Crithidia,* was used as a positive control. Wells containing cells without drugs were used as negative controls. A cell count of the culture was performed using a haemocytometer and the cell density was adjusted to the desired cell density. Usually, for trypanosomes, this would be 5×10^5 cells per ml. However, *Crithidia* has a faster growth rate than trypanosomes, reaching maximum density of 2×10^8 organisms per ml in 48 hours. Therefore, a density of 5×10^4 cells per ml was used. 100μl of cells were added to every well which reduces the drug concentration by half and therefore the highest drug concentration is 250μM. Due to the growth rate of *Crithidia,* the cells were incubated with the drugs for 7 hours instead of 48 hours, after which time 20 μl (10%) of Alamar Blue was added. If the cells are unaffected by the drug the dye will be metabolised causing a colour change from blue to pink. (See figure 8a). However, if the cells are sensitive to the drug the dye will remain blue. It was found that after the usual incubation period of 24 hours with the dye there was no colour change. The plates were incubated for a further 24 hours and an obvious colour change occurred which could be read on the automatic plate reader fluorometer.

Figure 5D: The Alamar Blue Assay

100μl of medium plus 100μl of test drug (A) in well. 100μl of cells is added. (B)

After incubation of cells with test drug for 7 hours 20μl of alamar blue is added.

After further incubation for 48 hours, living cells will metabolise the dye, changing the colour from blue to pink.
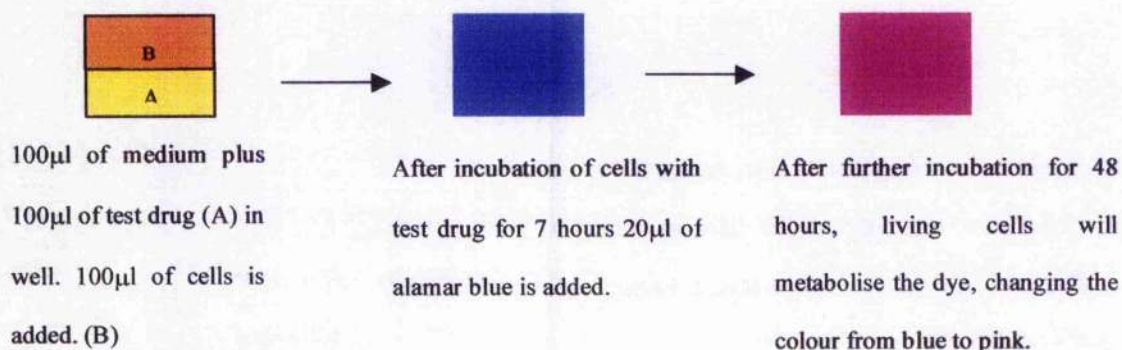
It is unclear why *Crithidia* requires an incubation time of 48 hours, as opposed to 24 hours, to metabolise the Alamar Blue. Reduction of Alamar Blue in trypanosomes is part of the glycolytic pathway (Raz et al, 1997) and therefore differences in this biochemical pathway, between the two organisms, may be responsible for the increase in incubation time essential to obtain a result.

Cell counts were also performed on individual wells to help validate the Alamar Blue assay as a reliable test for drug sensitivity in *C.fasciculata*. It was found that the cell counts corresponded to readings taken by the fluorometer. Viability of the cells was also noted.

RESULTS

Drug sensitivity of *C.fasciculata* to various trypanocidal compounds was examined using the modified Alamar Blue assay as described above. (Figure 5E)

Figure 5E

Drug sensitivity in *Crithidia fasciculata*

- pentamidine
- DMSO (20% in LIT medium)
- diminazene
- stillbamidine
- propamidine
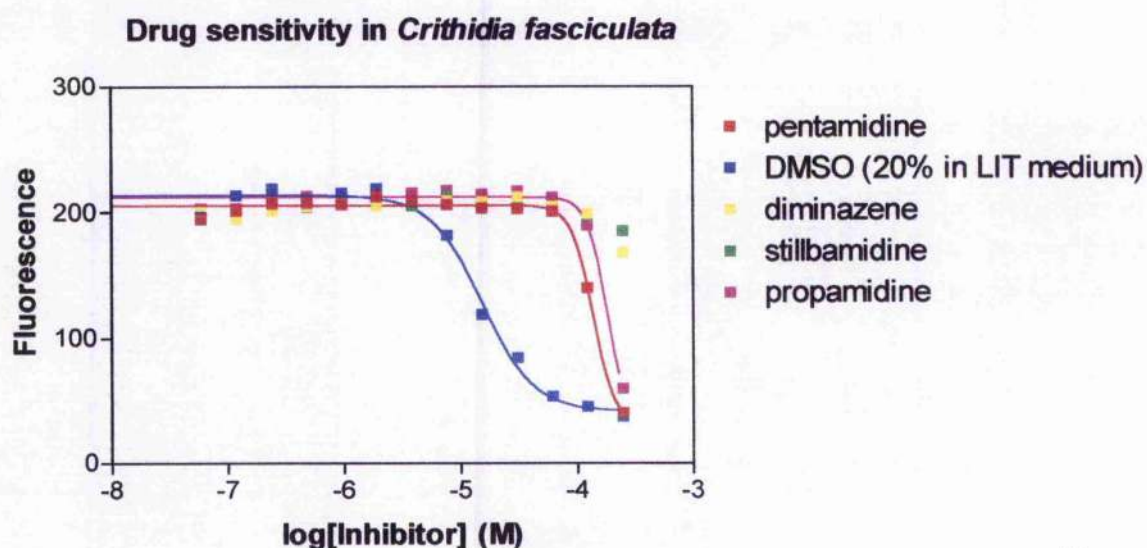
The results are plotted as measure fluorescence against the log concentration of test trypanocide. The graph shown is typical of the results found from three repeats. DMSO, as the positive control, kills all cells at the maximum concentration of 20%, which was confirmed microscopically. From the plot an IC_{50} of about 1.25 -2.5 % was calculated. It appears that pentamidine also kills cells at concentrations over 100 μM. However, this strain of *Crithidia* does not appear to be sensitive to concentrations lower than this and at 62.5 μM pentamidine has no effect. When examined microscopically cells were found to be fully motile in all wells including those containing the highest concentration of drug. However, the cell numbers were dramatically reduced, suggesting that pentamidine has an effect on the mechanisms involved in cell replication and division rather than a toxic effect leading to cell lysis.

Propamidine also had an effect on the cell growth at 250 μM but was less effective than pentamidine, having no effect on the cells at 125 μM and an estimated IC_{50} of about 181 μM compared to the lower IC_{50} of 138 μM for pentamidine (n =1).

C. fasciculata is not sensitive to diminazene, stillbamidine or butamidine (n = 4).

CHAPTER 6

Discussion

The structure–activity relationships of the pentamidine transporters HAPT1 and LAPT1

Several unlabelled potential substrates were used to inhibit the uptake of ^{3}H-pentamidine by HAPT1 and LAPT1 to gain an understanding of the structure-activity relationships of these transporters, to determine the individual specificities of each and to search for compounds that may be the true biological substrates present in the host environment. The structure-activity relationships between substrate and both HAPT1 and LAPT1, respectively appear to be extremely complex. The central alkane chain that joins the terminal polar groups, in diamidine compounds, is important in the binding and recognition of a substrate by HAPT1 and LAPT1. The part the chain plays in this mechanism is unclear. Variation of the chain length by the addition or subtraction of $(CH_2)_n$ groups has a dramatic effect on the ability of a diamidine compound to bind to the transporters. There is a clear correlation between the number of methylene groups and the affinity of a substrate for HAPT1 or LAPT1. The optimal chain length for binding is when n = 6-8 for LAPT1 and n = 5-6 for HAPT1. (Figure 3.7) Trypanocidal activity also increases as the length of the alkane chain increases to n =5. Beyond this the compounds become less active. (Lourie, 1939) This may be because as n increases the compound is taken up with a higher affinity and therefore the drug will be imported into the cell at a faster rate to show a higher activity towards the parasites. For HAPT1 and LAPT1, once the optimal chain length has been reached, affinity for the transporters appears to decrease as the chain length continues to increase.

It may be interesting to look for the same structure activity relationships between carbon chain length and binding to the P2 transporter. However, the carbon bonds in this chain are completely saturated rendering the chain inert and therefore it is unlikely that the actual chain is involved in binding to the transporter. It is possible that the ether links on either side of the chain are involved in binding, as they have free electron pairs that can engage in H-bonding. The chain length will affect the positioning of these groups. From another perspective, the apparent correlation between the affinity and the chain length may in fact reflect chain flexibility. The molecular model of pentamidine shows that it may not adopt a linear conformation under all conditions. It probably bends to co localise the two end charged groups, (Stead et al, 2001) particularly if stabilised in a complex. This is due to π-π orbital stacking of the benzene rings and allows these groups to share electrons and stabilises this conformation. At optimal orientation the binding energy of π-stackings by electrostatic and Van der Walls forces can reach up to 10 kJ. (Wallace et al, 2002, De Koning and Jarvis, 1999) Such a conformation will not be possible for very short or inflexible linking groups between the two diamidine rings.

If the alkane chain only acts as a linker between the outer rings and groups, then presumably, minor changes to the chain would not have a drastic effect on the affinity of the substrate for the transporter. In 1948, Schoenbach and Greenspan found that the introduction of two methyl groups to the alkane chain did not alter the trypanocidal activity but it is unknown if these modifications led to a reduced uptake of substrate. I found that by replacing the central chain with a furan ring decreased the affinity for both

transporters. The reason for this could be because the structure is more rigid which prevents the molecule from bending to allow π-π orbital stacking to occur. Alternatively, it may be due to a decrease in O atoms from two to one, which has led to a reduction in spare electrons available to form interactions with the binding pocket. The addition of methyl groups to the molecules increases the affinity and the position of these seems important, doubling the affinity for HAPT1 if moved from the benzene rings to the central furan ring. It appears that for high affinity binding, it is crucial that the middle portion of the diamidine remains as an alkane chain. The presence of two benzamidine groups appears to be essential for binding as demonstrated by the inability of benzamidine compounds to inhibit pentamidine uptake by HAPT1 and LAPT1. However, the addition of a hydroxyl group at position 4 on the benzene ring, promotes some binding activity to both HAPT1 and LAPT1. This group is equivalent to the ether oxygen in pentamidine. This shows the importance of an oxygen atom or other H-bond acceptor in the binding. The addition of large, bulky groups to position 3 on the benzene ring of pentamidine reduces binding activity possibly by physically preventing the binding.

The position of the side chain on the benzene ring, relative to the amide group, clearly affects binding to the transporter. This is illustrated most clearly by the difference in binding affinity between pentamidine and meta-pentamidine, but also by comparing 2-hydroxybenzamidine and 4-hydroxybenzamidine. It has been found that Berenil-resistant strains show no resistance to a modified drug with the amide groups at the meta position of the molecule (Hawking, 1963a). In the resistant line, the transporter may have

undergone a conformational change with the mutated transporter now selective for 'meta-Berenil'?

HAPT1 and LAPT1 appear to differ in ligand recognition profiles and therefore these binding pockets are distinguishable by their differences in specificity. The optimal chain length for binding to LAPT1 and HAPT1 differs, as previously discussed. Also, DAPI was shown to have a higher affinity for LAPT1 than the original substrate pentamidine, but a low affinity for HAPT1. It is unclear why this should be the case. However, in 1971 Dann et al showed that DAPI was slightly superior to Berenil in exerting trypanocidal action against *T.congolense*. DAPI is considered to have the same mode of action as Berenil, by binding to DNA and therefore these differences may be due to transport. Indeed Berenil displays little affinity for either HAPT1 or LAPT1 and it is unclear whether *T.congolense* expresses P2.

Another difference between HAPT1 and LAPT1 lie in their affinity for Berenil, isometamidium and ethidium bromide. Although both transporters have the highest affinity for isometamidium, HAPT1 has a higher affinity for Berenil than for ethidium bromide, whereas the opposite is true for LAPT1. Perhaps these transporters have slightly different biological functions and recognise different substrates from the host environment. It is possible that ethidium bromide and isometamidium are not only taken up by diffusion, as first thought, but by mediated transport through HAPT1 and/or LAPT1. It may yet appear that ethidium bromide only enters the cell via diffusion because little is transported by HAPT1 and none by the P2 transporter. Moreover, it is possible that other transporters with affinity for ethidium bromide exist and could be

involved in uptake. However, if LAPT1 does transport ethidium bromide, it is likely to play an important role in the transport of this drug, as it is a high capacity transporter, even for pentamidine for which it has a lower affinity (De Koning, 2001a ; Bray et al 2003) However, passive diffusion may still be the main mechanism of uptake.

It is unlikely that LAPT1 and HAPT1 play a part in the development of resistance to these drugs, as other mechanisms such as efflux pumps appear to be associated with isometamidium resistance (Sutherland et al, 1992a) There is little cross-resistance between isometamidium and Berenil and none between Berenil and ethidium bromide and resistance to each is thought to develop independently. This can be explained by a model in which these drugs are taken up by separate transporters: Berenil by P2 only and ISMM by HAPT1 and/or LAPT1.

There was one main problem encountered during these kinetic studies. In most of the experiments, unlabelled pentamidine was used as a control to show dose-dependant inhibition of the uptake of ^{3}H-pentamidine. However, at 4 mM LAPT1 never became fully saturated. It is thought that this could be for two reasons. The ^{3}H-pentamidine label was quite old and tritium on the pentamidine could have exchanged with water. This water may have been taken up into the cell, indistinguishable from ^{3}H-pentamidine and thereby causing an unsaturable background. It may also be possible that some pentamidine was entering the cells via diffusion, which is a non-saturable process, and is likely to occur at such high concentrations. This could be checked by using cells which are permeable to water but impermeable to pentamidine, such as erythrocytes.

Other general problems encountered when performing kinetic and transport studies involve the inability to directly measure the transport of substrates across the membrane. The K_i is a measurement of the competing substrates ability to block binding and therefore uptake of the radiolabelled permeant. As a molecule must bind and then travel across the membrane into the cell before the transporter can bind to another molecule, the time it takes for a substance to be physically transported across the membrane, affects the binding affinity of the transporter. A molecule with high affinity binding may take a longer period of time to cross the membrane, or vice versa, and this will make the K_i value higher or lower accordingly. However, studying transport in this way is considered to be standard, accurate and reliable.

The structure-activity relationships of HAPT1 and LAPT1 are obviously complicated and it is still unclear what the function of these transporters is in the host environment. It may be useful to look at pentamidine transport systems in similar organisms eg. kinetoplastids such as *Leishmania*. In 1976 Damper and Patton found that pentamidine did not share the same transport system as lysine and arginine in *T.brucei*. However, since then, pentamidine has been shown to inhibit arginine transport in *L.donovani* (Kandpal et al, 1995) and also shares the arginine/lysine transport system in *C.fasciculata*. It may be possible that HAPT1 and LAPT1 transport these substances. Pentamidine has also been shown to inhibit Ca^{2+} transport in *T.brucei brucei*. (Benain et al, 1993) and agents, which inhibit Ca2+ channels in *L.donovani*, were also shown to inhibit pentamidine uptake (Basselin et al, 2002) Perhaps HAPT1 and LAPT1 should be tested as Ca^{2+} transporters. Another idea is that HAPT1 or LAPT1 are polyamine carriers because a pentamidine

107

transporter in *L.donovani* has been shown to have this function and similarly is not sensitive to adenosine, like HAPT1 and LAPT1. (Basselin et al, 1996)

The P2 transporter as the sole mechanism for the uptake of Berenil.

From using ^{3}H-Berenil to directly measure the uptake of the drug, and using different well-known unlabelled substrates of P1 and P2 to inhibit this uptake, it is found that a single transporter is involved in the uptake of Berenil by *T.brucei* and this transporter has been identified as the P2 nucleoside transporter. The Ki values of known P2 substrates, pentamidine and adenosine, were 0.22 ± 0.02 µM and 0.25 ± 0.08 µM, respectively which are extremely close to the Ki values of these compounds for the P2 transporter which are 0.43 ± 0.02 and 0.92 ± 0.06 µM, respectively. There are several other pieces of evidence to support the results found in this study. Berenil has previously been shown to have no significant affinity for HAPT1 or LAPT1, which supports the finding that Berenil, enters the cell via one route only. (De Koning, 2000) As the drug is only taken up by the P2 transporter this provides an explanation for why there is a high level of cross-resistance between Berenil and melarsoprol in the field. The loss of P2 function would appear to confer resistance to both drugs. Although resistance to Berenil itself has been difficult to develop in the laboratory the loss of P2 function due to exposure to the veterinary arsenical drug cymelarsan may also lead to Berenil resistance and may account for the increase in Berenil-resistant cases as arsenical drugs become more widely used in animals. The low level of Berenil resistance in the field, compared to other veterinary drugs, is obviously not due to the presence of multiple Berenil transporters, as is probably

the case for related diamidine, pentamidine. It is possibly due to the drugs' rapid elimination from the host body, which renders the drug useless as a prophylactic.

In the past it has been speculated that *T.b.gambiense* or *T.b.rhodesiense*, infecting livestock, which are subsequently treated with subcurative doses of Berenil, could become resistant to the drug. If this strain, with a loss in P2 function, was then to be transmitted to a human the patient would also be refractory to treatment with melarsoprol. (De Koning, 2001) This worry is perhaps a little more justified now that there is conclusive evidence that Berenil is only taken up through the P2 transporter and presumably loss of function confers a drug resistant phenotype. This has been tested in the laboratory by inducing Berenil resistance and then monitoring for P2 function and activity (Barrett and Stewart, unpublished) and also in this study by using TbAT1 knockout cells, which lack a functional P2 transporter, to look at the affects on the kinetics of Berenil uptake.

It should be kept in mind that although there is only a single transporter for Berenil uptake in *T.brucei*, this might not be the case for other trypanosome species. Suswam, 2001, suggested that there was more than one Berenil transporter in *T.evansi* and yet it has been commented that *T.congolense* may not have a P2 transporter (Wilkes, unpublished) and if so how does the drug enter this trypanosome species? This is a relatively important question as *T.congolense* is the most prevalent species infecting cattle and is associated with almost all cases of Berenil resistance. The mechanisms of transport and resistance development may be completely different for this parasite, which

holds important implications for treatment regimes in the field and the development of new trypanocidal drugs.

The uptake of pentamidine by *Crithidia fasciculata.*

When *C.fasciculata* was incubated with 0.1µM of ^{3}H-Pentamidine over a period of 2 minutes, uptake of the drug was continuous and linear for the duration with no evidence of reaching a plateau. An increase in time points would probably show an increase in drug uptake until a maximum concentration had been accumulated. This may take as long as 24 hours as is seen in a pentamidine sensitive strain of *C.oncopelti.* (Wallis, 1966) At 1 mM there is no uptake because at this concentration the transporter is fully saturated. These results do confirm the presence of a pentamidine transport system with a relatively high affinity for pentamidine, which has a low Km value for the transporter at 0.66 ± 0.8 µM. When increasing concentrations of unlabelled pentamidine were used to inhibit the uptake of ^{3}H- Pentamidine the inhibition profile obtained shows that there is a single pentamidine transporter, which is saturable and conforms to Michaelis-Menten kinetics. However, the rate of pentamidine uptake is minimal and the Vmax is extremely low at 0.054 pmol (10^7 cells) $^{-1}$ s^{-1}) and probably the reason why Crithidia can accumulate pentamidine over a long period of time. Therefore, although a pentamidine transporter is present perhaps its contribution to the uptake of the drug is insignificant and elimination is not necessary for HS-6 to be used as an expression system for HAPT1 and LAPT1.

The toxicity results show that pentamidine does not have any effect on cells at concentrations below 62.5µM. Even at the highest concentration of 250µM there are still

motile cells present in the vials. This may be because the drug only has growth inhibitory effects on *Crithidia*, as previously suggested, (Newton and Le Page, 1967) or that this particular strain is partially resistant to pentamidine. Newton and Le Page also found that 17μM completely inhibited the growth of *C.fasciculata* in 12 hours and another study showed that 338μM inhibited all growth of cells after only 3 hours. (Wallis, 1966) Obviously, these parasites are much more sensitive to the drug than those used in this study but the reason for this is unclear. It has been previously suggested that perhaps pentamidine can enter the cells via the purine transport systems that have been identified. (Gutteridge, 1966) It has also been discovered that when cells are grown in purine free or purine depleted medium they increase the number of transporters in response to purine starvation (de Koning et al, 2000b) and accumulate purines at a faster rate. (Alleman, 1996, Hall et al, 1996) The same is true for several *Leishmania* species. (Seyfang and Landfear, 1999) This gives the parasite an advantage over the host to compete for the available nutrients. (Gero, 1997) Other parasites have also been shown to upregulate or downregulate the uptake of nutrients under stress. It may be possible that an excess of purines in the medium could cause the down regulation of purine transporters in *C.fasciculata* and if pentamidine enters through this system, organisms will have a reduced rate of uptake for the substrate and consequently the appearance of reduced sensitivity to the drug. It may be that the medium used here has slightly elevated levels of purines compared with the medium used to grow the parasites in previous experiments where the organisms appear to have a much greater sensitivity to pentamidine.

However, it has also been suggested that nutrient availability can directly affect the organisms' sensitivity to chemotherapeutic agents such as trypanocides. (Alleman, 1996).

A simple way to solve this dispute would be to identify the transport system involved in the uptake of pentamidine. By using the substrates of CfNT1 (adenosine) and CfNT2 (inosine and guanosine) to inhibit the uptake of pentamidine it can be determined whether the drug is competing with the natural substrates for these transporters.

Propamidine, a diamidine analogue closely resembling pentamidine, was used to inhibit the uptake of pentamidine and it was found to have a lower affinity for the same transporter with a Ki of 128.52µM (± 38.42). Propamidine also had a slight effect on cells in the Alamar Blue assay but only at the very high concentration of 250µM.

It is not surprising that propamidine is also taken up by this transporter as the biochemical structure is extremely similar to that of pentamidine and the transporter probably recognises a common motif on both compounds. It is also not surprising that propamidine has a lower affinity for the transporter, as this is what has been found for pentamidine transporters HAPT1 and LAPT1 in *T.brucei brucei*. However, there appears to be a correlation between the affinity for the transporter and the level of toxicity to cells. Pentamidine with a higher affinity for the transporter than propamidine also has a greater toxic effect on cells. Perhaps the lack of toxicity is due to the lack of sufficient uptake by the cells to cause either cell death or sufficiently inhibit cell growth. Wallis found that a pentamidine resistant strain also showed increased resistance to propamidine and stillbamidine. As it appears that this *C.fasciculata* strain may be partially resistant to pentamidine, perhaps this accounts for the lack of sensitivity to both these compounds.

A time course showed no evidence of ³H- Berenil uptake. However, this compound showed a high affinity for the pentamidine transporter by inhibiting the uptake of pentamidine. Rather than actually being transported across the membrane, Berenil is

possibly sticking to receptor sites on the transporter surface and therefore is blocking the uptake of ^{3}H- Pentamidine by preventing it from attaching to the transporter. This would suggest why there was no ^{3}H- Berenil found within the cell pellets. This would also explain why Berenil shows no toxic effects towards the organisms. However, this may also be due to a different drug target and mode of action compared to pentamidine, due to its more rigid structure. (Newton and Le Page, 1967) Wallis also found that his *C.fasciculata* strains were insensitive to Berenil.

The fact that this *C.fasciculata* strain has a pentamidine transporter, even though it may be insignificant, means that to gain more accurate results with the expression of HAPT1 and LAPT1, it must be knocked out. This is currently being achieved by exposing the organisms to increasing concentration of pentamidine and at present cells are beginning to grow well in a concentration of 500µM.

General Discussion

Considerable progress has been made towards the identification of the recognition motifs of HAPT1 and LAPT1. The fact that the transporters have different specificities for a variety of trypanocides suggests that they differ in biological function in the host environment, by mediating the uptake of separate substrates. However, it is still unclear what these substrates are. Presumably, they possess characteristics of the trypanocides that have been shown to have high affinity for HAPT1 and LAPT1 in this study. It is important to understand these structure-activity relationships more clearly in order to be able to predict other substances, which can enter the cell through these particular transport mechanisms. Substances found in the blood of the host could be randomly

tested for their affinity to HAPT1 or LAPT1, but this would be time consuming and so far has proved unsuccessful. The best hope is to characterise these transporters through using *Crithidia fasciculata* as an expression system. Once the sequences for the transporters are obtained, homology between genes in related organisms can be identified and perhaps provide an insight into the function of LAPT1 and HAPT1. With the increasing drug resistance that is associated with the loss of P2 function, it is important to find alternative drug targets or drug delivery systems in the parasite. HAPT1 and LAPT1 provide an alternative route for the uptake of trypanocides and therefore a drug with little affinity for P2 but a high affinity for HAPT1 and LAPT1 is of great value. On the other hand, if HAPT1 or LAPT1 transport a substance essential for the function of a biochemical or metabolic pathway, inhibitors could be used to block the uptake of such a compound and may, potentially lead to cell death. The identification of P2 as the transporter for Berenil has important implications for veterinary medicine. It is possible that Berenil-resistance is related to a loss of P2 function and, if so, there is no other way to deliver this drug into the cell, rendering it useless. The high levels of cross-resistance between arsenicals and Berenil in the field are worrying. Resistance to arsenical drugs is rapidly increasing and appears to be related to the loss of P2 function. Cymelarsan, a veterinary arsenical drug, is becoming more widely used and as arsenical resistance increases this will exacerbate the problem of Berenil resistance, which is the only veterinary drug left virtually unaffected by this disaster. The mechanisms for the uptake of several compounds have been identified in this study and a clearer overview now exists on the transport systems of *T.brucei brucei*. (Table 5)

This updated model of transporters in *T.brucei brucei* can be applied to human infective species T.b.gambiense and T.b.rhodesiense and may aid in the creation and the implementation of a new and more efficient treatment regime for both humans and cattle. It is now crucial that the function of HAPT1 and LAPT1 is identified for the design of novel chemotherapeutic agents in an attempt to combat the rapidly spreading drug resistance. However, whilst trypanosomiasis remains a neglected disease, in the funding sense, research progress will continue to be slow and there may not be much time left until the last effective trypanocide is rendered ineffective.

Table 5

Transporter	Substrate transported into the cell
P1	Inosine, Guanosine, Adenosine
P2	Adenosine, Adenine, Melaminophenyl arsenicals, Pentamidine, Diminazene
H2	Hypoxanthine, Adenine, Guanine, Guanosine
H3	Hypoxanthine, Adenine, Guanine
HAPT1	Pentamidine, Diamidines with increasing carbon chainlength from propamidine to octamidine, Isometamidium
LAPT1	Pentamidine, Diamidines hexamidine, heptamidine, octamidine, Isometamidium, Ethidium Bromide, DAPI.

An overview of the surface transporters involved in the uptake of trypanocides in *T.brucei brucei*.

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