The Role of the P2 Purine Transporter in Drug Uptake and Resistance in Trypanosomes

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Declaration

The results presented in this thesis are my own work, except where there is a statement to the contrary.

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Abstract

Human African Trypanosomiasis (HAT), caused by the parasitic trypanosomatids *T. b. gambiense* and *T. b. rhodesiense*, is endemic in over 36 countries in sub-Saharan Africa. Estimates place the numbers of infected individuals at around 500,000 with a further 60 million at risk. Treatment for HAT is available, although all the drugs in use have problems with availability, administration and disease stage specificity, and most cause very severe side effects. The arsenical based trypanocides are extremely toxic, causing death due to treatment in up to 10% of patients.

Reports of drug resistance in the field are wide spread. A few reports have suggested the involvement of the P2 transporter in the development of drug resistance. Point mutations within *TbAT1*, which encodes the P2 transporter, have been linked to drug resistant isolates in the field, and the loss of function of P2, through various molecular processes are a feature of laboratory derived drug resistant cell lines. However, the loss of P2 transport in a *ΔTbat1* cell line does not produce the high levels of resistance seen in laboratory derived drug resistant cell lines, and many isolates from relapse patients have shown no difference in *TbAT1* indicating that the situation in the field regarding resistance and P2 transport is very complicated, and that other routes to resistance exist. Nonetheless, an exponential increase in the prevalence and distribution of HAT, coupled with an increased incidence of reported drug resistance, makes the development of new trypanotoxic compounds vital.

The exploitation of the P2 purine transporter as a parasite-specific entry point for new trypanocidal compounds has been investigated in this thesis. The P2 transporter is ideal for this approach to drug design due to its high affinity for substrates carrying the primary recognition motif, $H_2N\text{-}C(R_1)=N\text{-}R_2$, the lack of any similar motif on mammalian purine transporters and its natural substrates being at very low concentrations within the bloodstream of the host. Melamine and benzamidine groups, which carry the recognition motif for the P2 transporter, were attached to potentially cytotoxic moieties (polyamine analogues, HIV gp120 binding mimetics, nitrofuran and nitroimidazole compounds) in order to target them specifically to the parasite transporter and hence the parasite.

All compounds carrying a melamine or benzamidine group were able to block the uptake of radiolabelled adenosine (a natural substrate of P2) to some degree. Toxicity varied, but some compounds were extremely trypanotoxic *in vitro*, and one nitrofuran is active *in vivo* and could represent a promising lead compound. *In vitro* toxicity experiments using a *ΔTbat1* (P2 knockout) cell line, reveal no innate resistance to P2 recognition motif bearing compounds in these cells, suggesting that the P2 transporter is not crucial for the toxic actions of these compounds, allaying fears over the development of resistance and cross resistance to other trypanocides through the loss of P2.

The role of the P2 transporter in the development of drug resistance was investigated in a *T. equiperdum* cell line (P) and a Berenil resistant cell line (PBR) derived from it that had been reported to have a P2 transport component with greatly reduced affinity for adenosine. Sequencing results indicate that *TbAT1* in *T. equiperdum* P exists as two alleles, whereas the *T. equiperdum* PBR cell line only contains sequence corresponding to one. Additionally, the *T. equiperdum* PBR cell line lacks any *TbAT1* transcript detectable by Northern blot, which suggests that this loss of heterozygosity in *T. equiperdum* PBR has resulted in a lack of transcription of the gene, and a loss of P2 transport activity. An adenine sensitive adenosine transport component was identifiable in this cell line as well as in the *ΔTbat1* cell line, suggesting the presence of an, as yet unidentified, low affinity adenosine transporter in trypanosomes.

Finally, as loss of P2 has been seen to contribute to drug resistance in both laboratory derived cell lines and field isolates, a quick and simple test has been developed that is capable of identifying cells lacking the P2 transporter, and are therefore tolerant to pharmacologically relevant levels of diamidine and arsenical trypanocides. This simple test could be adapted for use in the field either for research purposes, or by clinical staff, with the aim of identifying patients with drug resistant trypanosomiasis, and tailoring their treatment accordingly.

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List of Abbreviations

ATP	Adenosine triphosphate
Ado-S-Me	methylthioadenosine
BBB	Blood Brain Barrier
BoTat	Bordeaux trypanozoon antigenic type
bp	Basepair
CBSS	Carter's Balanced Salt Solution
CD_{100}	100%Curative
Ci	Curie
CNS	Central Nervous System
CSF	Cerebospinal fluid
DAPI	4',6-Diamidino-2-phenylindole
dAdoMet	decarboxylated S-adenosylmethionine
dATP	deoxy adenosine triphosphate
dCTP	deoxy cytosine triphosphate
dGTP	deoxy guanine triphosphate
dTTP	deoxy thymidine triphosphate
DEAE	diethyl-amine-ethyl
DFMO	Diflouromethylornithine
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DRC	Democratic Republic of Congo
EATRO	East African Trypanosomiasis Research Organisation
EDTA	Ethylenediamine tetraacetic acid
g	gram
GSH	glutathione
h	hour
HAPT1	High Affinity Pentamidine Transporter
HAT	Human African Trypanosomiasis
hsDNA	herring sperm DNA
IC_{50}	50% inhibitory concentration
ICR	Institute for Cancer Research
IPTG	isopropylthio-β-D-galactoside
kb	kilobase
kg	kilogram
K_i	Inhibition constant
kJ	kilojoule
K_m	Michaelis constant
LAPT1	Low Affinity Pentamidine Transporter
LDL	Low Density Lipoprotein
ls	long slender
Mb	Megabase
MEC_{100}	100% Minimum Effective Concentration
mg	milligram
ml	millilitre
mM	millimole
mmol	millimolar
mV	millivolt
nm	nanometer
ng	nanogram

ODC	Ornithine Decarboxylase
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
Pfu	*Pyrococcus furiosus*
pmol	picomole
PNP	Purine Nucleoside Phosphorylase
PSG	Phosphate buffered Saline, 1% Glucose
RFLP	Restriction enzyme Fragment Length Polymorphism
RIME	Ribosomal Inserted Mobile Element
RNA	Ribonucleic acid
rpm	revolutions per minute
s	second
SDS	Sodium Dodecylsulphate
SRA	serum resistance associated
ss	short stumpy
SSC	Saline Sodium Citrate
SSCP	Single Stranded Conformational Polymorphism
STIB	Swiss Tropical Institute, Brunel
Taq	*Thermus aquaticus*
TbAT1	*T. brucei* Adenosine Transporter
TbNBT1	*T. brucei* Nucleobase Transporter
TIM	Triosephosphate isomerase
U	Unit
UV	Ultraviolet
V	Volt
V_{max}	Maximum Velocity
VSG	Variant Surface Glycoprotein
WHO	World Health Organisation
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
μM	micromolar
μl	microlitre

Chapter I

Introduction

1.1 Current Situation of Human African Trypanosomiasis (HAT)

At the beginning of the 1900's the incidence of HAT was extremely high. One review places the number of human cases of trypanosomiasis at over 1 million, with over 250,000 reported deaths in Uganda (Seed 2000). By the 1960's and early 1970's this figure was greatly reduced due to the use of insect vector control strategies (use of insecticides and clearing of vegetation), the removal of animal reservoirs (by hunting and culling) and the screening and treatment of infected people. The number of infected individuals was placed at around 10,000 – 20,000 during this period.

Due to the lack of screening and diminished use of insecticides targeted against the tsetse fly this number has risen over the intervening years to the present day situation where approximately 60 million people live in endemic areas in 36 countries in sub-Saharan Africa (Barrett 1999). 200 discrete endemic foci have been described, and epidemics have emerged in Sudan, Uganda, Angola and the Democratic Republic of Congo (DRC), (Figure 1.1). 36,000 cases of HAT were reported in 1998, however, as only 5-10 % of the population was screened the figure is considered to be closer to, and more likely greater than, 300,000 persons infected in that year (WHO, Report of a WHO Expert Committee, 1998).

The current epidemics reported in Angola, the DRC, southern Sudan and Uganda are mostly due to infection by *T. b. gambiense*. In the DRC in 1977 there were 3,818 new cases reported per year. This rose to 19,340 new cases reported in 1994, according to World Heath Organisation figures (WHO, Report of a WHO Expert Committee, 1998). Just as dramatic is the rise in reported new cases in Uganda from 52 in 1970, to more than 1,500 in 1994. The prevalence of infection in southern Sudan rose by 20 %, and areas of Angola now have a prevalence of 60 %. Many of the epidemic areas are inaccessible due to war, and up to 50 % of those infected are in the secondary stage of HAT (Legros et al. 2002; Seed 2000).

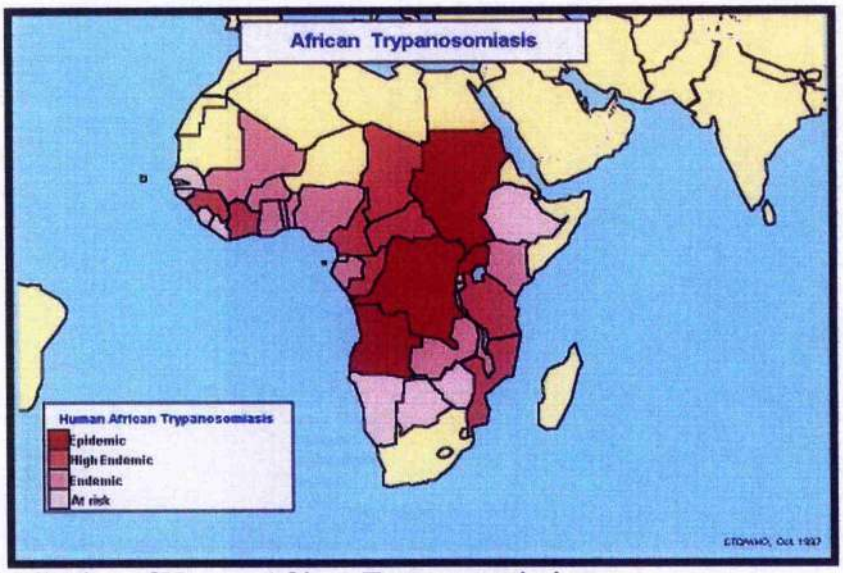


Figure 1.1 Distribution of Human African Trypanosomiasis.
Reproduced from http://www.who.int/emc/diseases/tryp/trypanogeo.html

War in an endemic area can increase the spread of trypanosomiasis, especially the gambiense form of the disease. The primary stage of gambiense sleeping sickness can last for many months, even years, with just general symptoms of malaise. In this time an infected person can act as a walking reservoir, capable of spreading disease. Many refugees migrating from war-torn epidemic areas carry trypanosomiasis with them to new areas where tsetse flies native to this new environment spread the infection. The outbreaks in Uganda, for example, are partly due to the migrations of peoples out of southern Sudan for this reason (Kuzoe 1993). This exponential increase in the prevalence and distribution of HAT, coupled with an increased incidence of reported drug resistance, has sparked an increase in research activity concerned with the discovery of new treatments for HAT.

1.2 The Trypanosome

1.2.1 Taxonomy

Trypanosomes are protozoons of the order Kinetoplastida, family Trypanosomatidae. Trypanosomatids include the genus *Leishmania*, species and subspecies of which are

responsible for human diseases such as visceral leishmaniasis (*L. donovani*), cutaneous and mucocutaneous leishmaniasis (*L. braziliensis*), and diffuse cutaneous leishmaniasis (*L. mexicana*). The genus *Trypanosoma* can be further divided into two groups of infective parasites, the stercoraria and the salivaria. The stercoraria include the human infective species *T. cruzi*, the causative agent of South American trypanosomiasis. The human infective *T. brucei gambiense* and *T. brucei rhodesiense*, are salivarian parasites of the subgenus *Trypanozoon* (Figure 1.2), which also contains the animal infective species *T. equiperdum*, *T. evansi* and *T. brucei*.

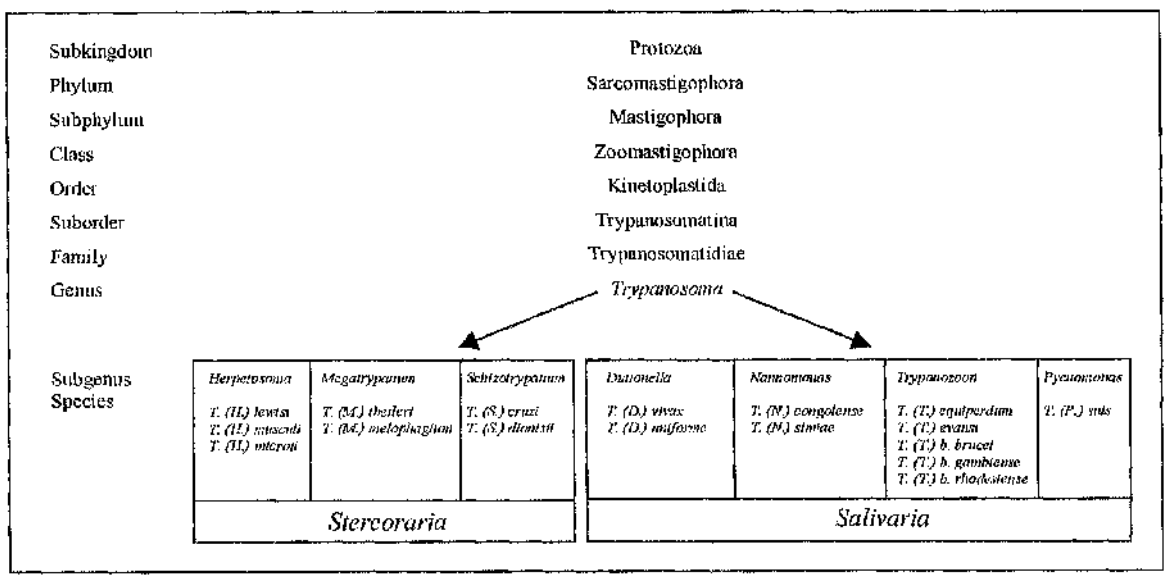


Figure 1.2 Classification of Trypanosomes.
From WHO, Report of a WHO Expert Committee, 1998.

T. brucei is regarded as a collection of subspecies which include the human infective species and the animal infective *T. b. brucei*. These three subspecies are morphologically indistinguishable, but can be distinguished by isoenzyme analysis and restriction enzyme fragment length polymorphism (RFLP) (Gibson 2002; Kanmogne et al. 1996; Truc & Tibayrenc 1993).

T. b. rhodesiense and *T. b. brucei* are closely related, but *T. b. rhodesiense* are differentiated by their infectivity for humans. Rhodesiense parasites are able to withstand lysis by human serum due to the action of a serum resistance associated (SRA) gene (De Greef et al. 1992) expressed from a variant surface glycoprotein gene expression site (Hager & Hajduk 1997;

Milner & Hajduk 1999). Human infective trypanosomes can be detected in animal reservoirs by identification of the SRA gene (Welburn et al. 2001). *T. b. rhodesiense* and *T. b. brucei* can also be differentiated by minisatellite marker analysis (MacLeod et al. 2000).

T. b. gambiense is characterised as a distinct group, but can be further divided into two categories based on the clinical presentation of the disease. The Type I group causes a disease pattern that shows classical, chronic, *T. b. gambiense*-like progression and Type II causes a more acute disease which is more like that caused by *T. b. rhodesiense* in development. Type II parasites give a negative result on polymerase chain reaction (PCR) analysis of the variant surface glycoprotein (VSG) gene Antat 11.17 (Bromidge et al. 1993), as did *T. b. rhodesiense* and *T. b. brucei,* and have shown different RFLP patterns to Type I cell lines (Agbo et al. 2001).

1.2.2 Life Cycle

Figure 1.3 illustrates the life cycle of *T. b. brucei, T. b. gambiense* and *T. b. rhodesiense,* all of which are transmitted by an insect vector, the tsetse fly.

A fly becomes infected when it takes a blood meal from an infected host. Within the insect's midgut, the pre-adapted short-stumpy forms differentiate into elongated procyclic trypanosomes. These procyclic cells further differentiate in the hindgut into mesocyclic cells, which then migrate to the oesophagus and the mouthparts of the insect, eventually travelling up the salivary ducts to the salivary gland. In the salivary gland they proliferate rapidly as the epimastigote forms which are attached to the walls of the salivary gland by a flagellipodium, a dendritic outgrowth of the flagellum. Epimastigotes mature into the free living metacyclic forms, which are pre-adapted for life within the mammalian host. Metacyclic forms are then introduced into the mammalian bloodstream through the bite of the fly and undergo differentiation into the long slender bloodstream form (Vickerman 1985). The long slender form of the parasite divides within the mammalian bloodstream and undergoes antigenic variation (Turner 1999).

Antigenic variation refers to the ability of the population of infecting cells to change its major surface glycoprotein in order to evade the host's immune system. The metacyclic cells that are introduced into the bloodstream are a mixed population, carrying different VSGs. As these cells differentiate into long slender bloodstream forms and divide, the host's immune system is able to identify and mount a response to the predominant VSGs. At the same time a small subpopulation switch their dominant surface antigen. Consequently, as the older populations of cells are eradicated, others are able to grow up before an immune response is mounted against them. As this process is repeated, a population of trypanosomes is able to evade the host's immune system, and present a fluctuating parasitaemia within the bloodstream (Barry & McCulloch 2001).

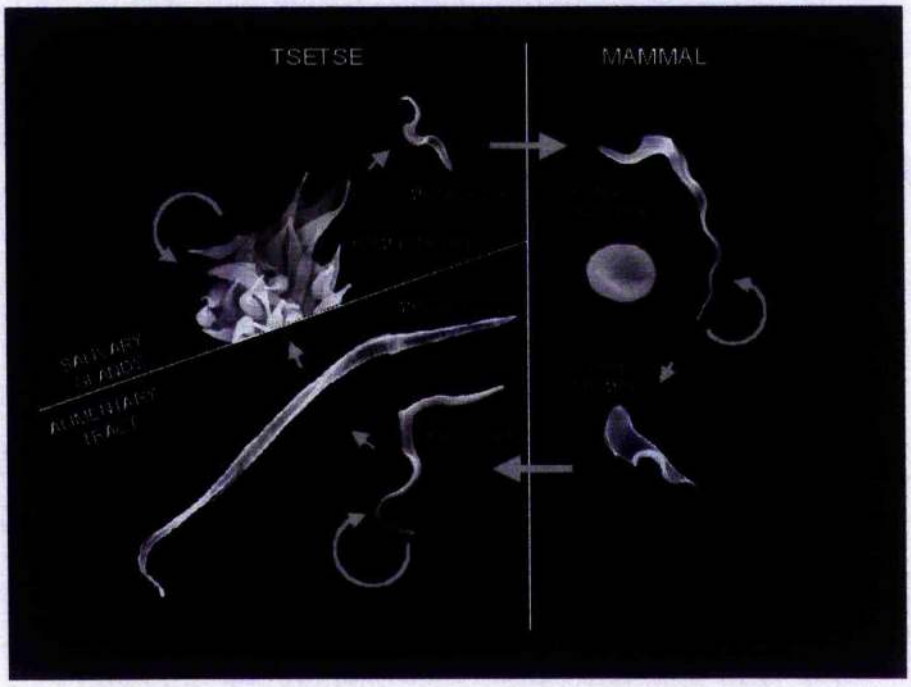


Figure 1.3 Life cycle of *Trypanosoma brucei.*
Illustration constructed by D, Barry, University of Glasgow.

When a population of long slender forms reaches a peak parasitaemia some of the cells differentiate into short stumpy forms. These cells are non-proliferative and are quickly eradicated from the mammalian host's bloodstream, but are pre-adapted to continue the cycle

of infection in the tsetse fly vector, whereas the proliferative long slender forms are destroyed in the midgut (Matthews 1999).

T. b. gambiense and *T. b. rhodesiense* are ordinarily transmitted by different species of tsetse fly, varying in their habitats. *T. b. gambiense* is transmitted by *Glossina palpalis* and other related species which normally live along river banks. *T. b. rhodesiense* is transmitted by *Glossina morsitans* and related species, which are savannah dwelling species (WHO, World Health Report, 2002). Although their ranges can overlap, the nature of the vector species largely determines the distribution of the disease.

Other parasitic trypanosomes infective to mammals do not undergo cyclical transmission through an insect. *T. evansi* is a parasite of wild and domestic animals that has lost the ability to pass cyclically through an insect vector. Infections are carried to a new host by mechanical transmission on the mouthparts of biting insects. *T. equiperdum* has specialised even further and only infects equines and has no need of any insect 'carrier', but is instead transmitted during coitus (Stephen 1986).

1.2.3 The Trypanosome Genome

Trypanosomes possess three different classes of nuclear chromosomes, the megabase chromosomes (1 Mb - >6 Mb), the intermediate chromosomes (200-900 kb) and the minichromosomes (50-150 kb). These chromosomes have been separated and identified by pulsed field gel electrophoresis as the chromosomes do not condense during mitosis. The haploid DNA content is approximately 35 Mb with up to 25 % variation between strains (El Sayed et al. 2000).

There are 11 pairs of megabase chromosomes, numbered I to XI according to their size. They are diploid, but homologous pairs are often different sizes. Southern hybridisation with various probes suggests that they carry a bloodstream form VSG expression site at one end, and a metacyclic VSG expression site at the other (El Sayed et al. 2000). Only one VSG expression site is active at any one time. The mechanisms for silencing the remaining sites, and for changing expression to other sites are not known, although the recent identification of an RNA

polymerase I body, independent of the nucleolus, that associates with active expression sites might be of significance (Navarro & Gull 2001). Megabase chromosomes also carry housekeeping and trypanosome specific genes on their interior as well as large regions to which ribosomal inserted mobile elements (RIME) and *ingi* probes hybridise. These *ingi*/RIME regions vary in size and contribute greatly to the size polymorphism in megabase chromosomes (El Sayed et al. 2000).

Intermediate chromosomes vary in number and size, and their ploidy is uncertain. They contain few unique markers, but can carry telomere linked VSGs. It is possible that they act as a repository for VSG genes or VSG expression sites (El Sayed et al. 2000).

Minichromosomes may also provide a store for inactive VSG genes, as no other function has yet been identified for them. Up to 90 % of the chromosome consists of 177 bp repeats, which are separated from the telomeres by GC and AT rich repeats. Although some telomeres are linked to silent VSG expression sites, no active site on a minichromosome has been identified so far (Barry & McCulloch 2001).

In addition to their nuclear DNA, trypanosomes possess an interlinked network of DNA in their mitochondria, the kinetoplast, which consists of supercoiled minicircles (~ 1 kb) and maxicircles (~ 20 kb) and make up to 20 % of a trypanosome's total DNA content. The maxicircles are the equivalent of mitochondrial DNA in other eukaryotic organisms, and the minicircles encode a series of guide RNAs. These serve as templates for the insertion and deletion of uridine residues in the maxicircle transcripts, a process known as RNA editing (Benne 1994). These modifications can be on a large scale, with more than half of the nucleotides in a reading frame being added by this process (Feagin et al. 1988).

The sequencing of the *T. brucei* genome is underway. There are at least ten laboratories world-wide contributing to this work, co-ordinated by the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Disease (TDR). This network aims to characterise further the *T. brucei* genome and provide an integrated facility open to all researchers working on *T. brucei*. The genome is considered to be around 90 % complete at the time of writing, with an expected completion date in 2004.

1.3 Disease, Diagnosis and Treatment

1.3.1 Clinical Disease

HAT has two clinical phases, distinguished by the presence, or absence, of parasites within the central nervous system (CNS). The first stage of the disease is essentially a haemolymphatic phase where the parasites reside within the blood and lymphatic systems, as well as the extra cellular spaces outside of the CNS. Second stage disease occurs when trypanosomes invade the CNS establishing a meningoencephalitic infection (Enanga et al. 2002). *T. b. gambiense* and *T. b. rhodesiense* infections display somewhat similar symptoms, but the rhodesiense disease progresses much faster than the gambiense form, progressing to second stage disease and death within months or even weeks. Gambiense infections, on the other hand, can remain quiescent for many months, even years (WHO, Report of a WHO Expert Committee, 1998).

First stage disease presents no more specific symptoms than fever, severe headaches and joint and muscle pain. A chancre can develop at the site of the tsetse fly bite, and lymphadenopathy can occur. In gambiense disease this is often confined to the posterior cervical lymph nodes (Winterbottom's sign), but in rhodesiense disease it is more generalised. After the trypanosomes migrate through the blood-brain barrier, neurological symptoms become apparent. This second stage of the disease can manifest itself in sleep disturbances (hence the common name 'sleeping sickness'), confusion and disorientation and personality and behavioural changes. If left to develop further, there is a progressive loss of consciousness, which culminates in coma and death (WHO, Report of a WHO Expert Committee, 1998).

1.3.2 Diagnosis

Positive diagnosis of the disease, at all stages, is dependent on the identification of trypanosomes within the blood, lymphatic fluids or cerebrospinal fluids of patients. Parasitaemia can be extremely low, so concentration techniques are often used to help view the parasites. As the very early symptoms of the disease are not normally severe, and health care in most endemic areas is inadequate and sparse, patients are very rarely diagnosed in the

early stages of infection. As a consequence most patients present themselves in the later stages of the disease when the parasite has already invaded the central nervous system (CNS).

That parasites have invaded the CNS, and the disease has progressed to the second stage, can be established by examining a sample of cerebrospinal fluid for trypanosomes. These can be at very low levels, and difficult to detect, so the presence of above normal numbers of leukocytes (> 5 cells/ml) within the CNS or high protein levels (> 37 mg/100 ml) is used as an indicator of CNS involvement (WHO, Report of a WHO Expert Committee, 1998).

1.3.3 Treatment

The drugs used to treat HAT are disease stage specific. Drugs used against the later stage of the disease can be very toxic or difficult to obtain and administer, and those used against the primary stage are ineffective against infections that have already invaded the CNS. Because of this, it is important to determine at what stage of disease the patient is presenting before treatment begins.

1.3.3.1 Treatment of Early Stage Disease

Suramin (Figure 1.4), which was first used in 1922 (Voogd et al. 1993), can be used to treat both gambiense and rhodesiense early stage infections. At physiological pH, Suramin, a polysulphonated naphthalene derivative, is highly charged with 6 negative charges. The drug binds to many serum components, including low density lipoprotein (LDL) for which trypanosomes have a receptor (Vansterkenburg et al. 1993), and uptake has been reported as being mediated by LDL bound drug being internalised by receptor mediated endocytosis. A recent report has, however, reported a non-LDL receptor mediated uptake component in trypanosomes (Pal et al. 2002). A mode of action has not been deduced, but seems likely to be related to its highly charged state mediating electrostatic interactions with target molecules.

Pentamidine (Figure 1.4), an aromatic diamidine, has been used to treat early stage *T. b. gambiense* infections for over fifty years (Sands et al. 1985). The drug is concentrated to millimolar levels within the trypanosomes due to its accumulation through active transport (Carter et al. 1995). Pentamidine is taken up through the P2, LAPT1 and HAPT1 transporters

(see later). As a polycation, pentamidine interacts with anionic molecules within the trypanosome, including DNA (Wang 1995). Other targets have been suggested, but none confirmed.

Treatment of trypanosomiasis with pentamidine requires 7 daily intramuscular or intravenous injections of 4 mg/kg. The drug can cause serious side effects including hypotension, nausea, nephrotoxicity and can result in diabetes.

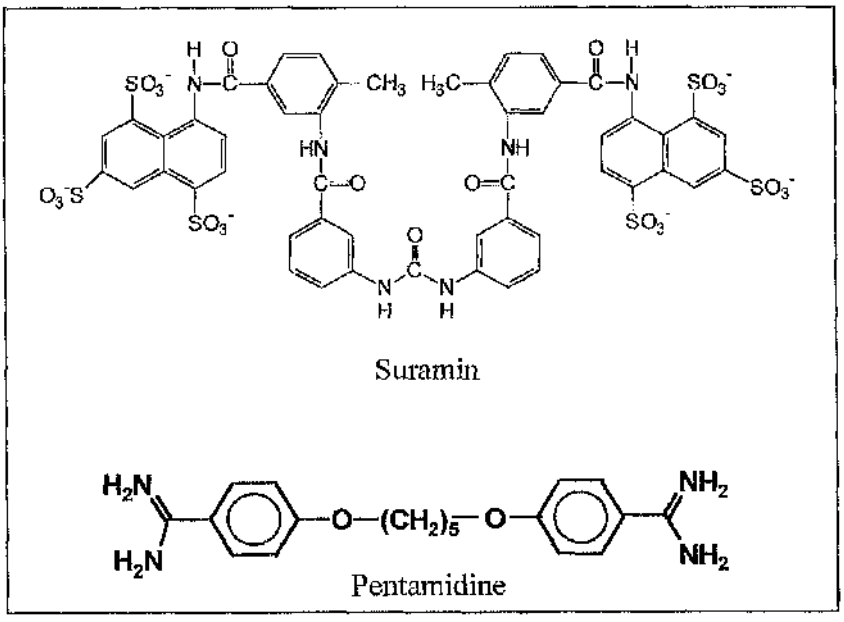


Figure 1.4. Drugs used for the treatment of early stage disease.

1.3.3.2 Treatment of Late Stage Disease

Second stage rhodesiense and gambiense disease is most commonly treated with melarsoprol (Figure 1.5), a melaminophenyl arsenical that was introduced in 1949. Melarsoprol itself is lipophilic and will enter cells via passive diffusion. It is able to diffuse across the blood brain barrier (BBB), and clear CNS infections. However, melarsoprol is readily converted to melarsen oxide (Keiser & Burri 2000), which is less able to cross the BBB. Melarsoprol and melarsen oxide are also accumulated in the trypanosome cell by the P2 purine transporter, the

loss of function of which has been associated with resistance to arsenical and diamidine drugs (see later).

Several modes of action have been suggested for melarsoprol, including inhibition of enzymes of glycolysis (Denise et al. 1999; Van Schaftingen et al. 1987) and interactions with low molecular weight thiols (Fairlamb et al. 1989; Fairlamb et al. 1992a). However, rapid lysis of trypanosomes has been shown to occur before either of these interactions have occurred to levels likely to elicit a toxic effect on the cells (Fairlamb et al. 1992a; Van Schaftingen et al. 1987).

As a trivalent organic arsenical melarsoprol is toxic, requiring hospitalisation for treatment and causing many, often severe, side effects. The most severe reaction to treatment is a reactive encephalopathy occurring in up to 10 % of patients, which can lead to death in up to 5 % of those treated.

In 1990 Eflornithine (difluoromethylornithine, DFMO, Figure 1.5), was licensed for use. This compound is very successful in treating both early and late stage *T. b. gambiense* with very few side effects, although *T. b. rhodesiense* cells have an innate resistance to the trypanotoxic effects of the compound. DFMO is the only trypanotoxic compound for which a mechanism of action is known. It is an inhibitor of ornithine decarboxylase (ODC), and as such was developed for the treatment of cancer, but not carried forward into therapeutic use (Barrett & Barrett 2000). Its selective toxicity for the parasite stems from the low rate of turnover of ODC in *T. b. gambiense* as compared to that in mammalian cells (Phillips et al. 1987). The effect of DFMO on trypanosomes is cytostatic in nature, requiring an active host immune system to clear infections (Bitonti et al. 1986b). Treated cells appear to be more like the short, stumpy forms in the bloodstream and are non-dividing and incapable of changing their VSG coat.

The activity of DFMO against trypanosomes may be due to a number of factors. The decrease in levels of putrescine and spermidine will cause a disruption of many metabolic processes, and in particular a decrease in trypanothione levels and an increase in decarboxylated

s-adenosylmethionine may contribute. A decrease in the levels of trypanothione in the cells would leave them vulnerable to oxidative damage (Fairlamb et al. 1987).

DFMO is reported to enter the cell by passive diffusion (Bitonti et al. 1986a), although one report notes the presence of a saturable transport process (Phillips & Wang 1987). The treatment regimen of 4 times 100 mg/kg doses, daily for 14 days requires a great deal of the compound, which is costly to produce and administer, requiring hospitalisation. Moreover the continued production of DFMO is not secured which contributes to the unsatisfactory status of this agent as a trypanocide.

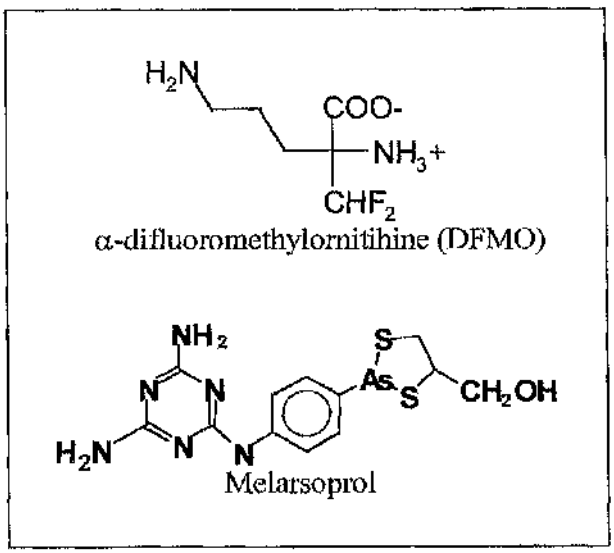


Figure 1.5. Drugs used for the treatment of late stage disease.

1.4 Drug Resistance

All of the above compounds have problems in their administration or toxicity to patients. Their availability and stability is an issue in affected areas where health care is minimal, if present at all. In addition, reports of treatment failures with these drugs, many of which have now been used for over fifty years, are emerging in the field.

In a gambiensian disease focus in Uganda, 30 % of patients treated with melarsoprol relapsed within two years of treatment (Legros et al. 1999). In an Angolan focus, 25 % of patients were

seen to relapse within 30 days (Stanghellini & Josenando 2001), and reports of 16 – 21 % relapse rates have been reported in Angola (Brun et al. 2001).

Among the most common reasons for relapses from drug therapy are poor dosing regimens, differing drug pharmacokinetics between patients (*e.g.* inability to cross the BBB in sufficient quantities), or factors presented by the parasites themselves leading to an increased tolerance to drugs. That all late stage treatment is administered in hospitals, under the supervision of trained medical staff, makes it unlikely that poor treatment regimens are involved in relapses. Relapse caused by a decreased ability for drug to cross the BBB in some patients is likewise seen as unlikely. A study undertaken in an area of high relapse rates in Uganda has shown that the levels of melarsoprol available in the CNS of patients recovering after a single treatment, and those that have relapsed, reach the same levels. Consequently, relapse is most likely to result from changes within the parasite itself leading to an innate tolerance, or resistance to trypanocides. However, it has been difficult to show significant differences in susceptibility of parasites isolated from cured and relapse patients (Brun et al. 2001).

Resistance to the first stage treatments, suramin and pentamidine, is not considered to be a serious risk at present. Moreover, any reported treatment failures could be due to the misdiagnosis of early-late stage infections as early stage infection, and the subsequent reinvasion of trypanosomes from the CNS.

The innate resistance shown by *T. b. rhodesiense* to the late stage compound, eflornithine, which is effective against *T. b. gambiense*, has been demonstrated to be due to a difference in the rates of turnover of its target, ornithine decarboxylase. Just as the lack of toxicity to mammalian cells is due to the rapid turnover of mammalian ODC as compared to that of *T. b. gambiense*, *T. b. rhodesiense* ODC has a higher specific activity and a shorter half life (4.3 h) than its gambiensian equivalent (>18 h) (Iten et al. 1997).

Resistance to melarsoprol has been readily induced in laboratory strains, and is now being seen in the field. The development of resistance to arsenicals, and cross-resistance to diamidines, has been proposed to be mediated by the loss of function of the adenosine transporter, P2 (Carter & Fairlamb 1993).

By screening a range of nitrogenous biochemicals, Carter and Fairlamb demonstrated that the lysis of trypanosomes by melarsen oxide was delayed by the addition of adenosine to lysis assay buffer. They also demonstrated that the transport of [^{3}H]adenosine into trypanosomes could be inhibited by melarsen oxide, suggesting that an adenosine transporter was involved in the uptake of this drug into trypanosomes. Further studies showed there are two distinct adenosine transport systems, one inhibitable by inosine, accounting for around 70 % of adenosine uptake, and one inhibitable by adenine, melarsoprol and melarsen oxide, accounting for approximately 30 % of adenosine uptake. The two transport systems were labelled P1 and P2 respectively, and a melarsen resistant line, *T. b. brucei* 427 cRU15 (Fairlamb et al. 1992b), was shown to have a reduced P1 activity and to have lost P2 activity altogether (Carter & Fairlamb 1993).

This same melarsen resistant strain was also resistant to some diamidines. Pentamidine blocked the lytic action of melarsen oxide as well as inhibiting the uptake of adenosine, showing an interaction with P2. However, adenosine failed to block the uptake of radiolabelled pentamidine to the expected degree. A 100 fold excess of adenosine could only block pentamidine uptake by 85 %, indicating that additional transporters for pentamidine could play a role in its uptake (Carter et al. 1995). The presence of additional transporters for pentamidine has since been confirmed with a further two transporters identified in *T. b. brucei* (de Koning 2001b).

1.4.1 The Molecular Basis of Drug Resistance

With the emergence of drug resistant and cross-resistant strains, the search for the molecular basis of resistance has become more important. The gene encoding the P2 nucleoside transporter, *TbAT1*, was described by Mäser from a *Trypanosoma brucei brucei* library transfected into a strain of yeast defective in purine biosynthesis (Mäser et al. 1999). The transfected yeast cell line, which does not normally transport adenosine, was then plated out onto a medium containing adenosine as the sole source of purine. In this way the transfected cells, which contained *TbAT1* encoding the *T. brucei* P2 transporter, were isolated.

TbAT1 encodes a 463 amino acid hydrophobic polypeptide, with 11 predicted transmembrane α-helices, a cytosolic amino terminus and an extracellular carboxy terminus and a large cytosolic loop between transmembrane domains 6 and 7 (Figure 1.6). The P2 transporter expressed in yeast was inhibitable by adenine and made transfected cells more susceptible to arsenicals than wild type cells. Transport studies showed, however, that pentamidine was unable to block adenosine uptake in the cells, which could be due to incorrect processing of the transporter protein in yeast cells.

The P2 transporters from an arsenical resistant strain, STIB 777R and the arsenical sensitive parent strain, STIB 777S, were then cloned and expressed in yeast. The sequence from the sensitive strain was found to be identical to the sequence already described, but the sequence of the resistant strain had 10 nucleotide changes that translated into 6 changes at the amino acid level (Figure 1.6). The resistant clone expressed in yeast failed to increase sensitivity to arsenicals, and did not induce uptake of adenosine either, suggesting that the mutation in STIB 777R has produced a non-functional P2 transporter, as opposed to one that has altered substrate specificity.

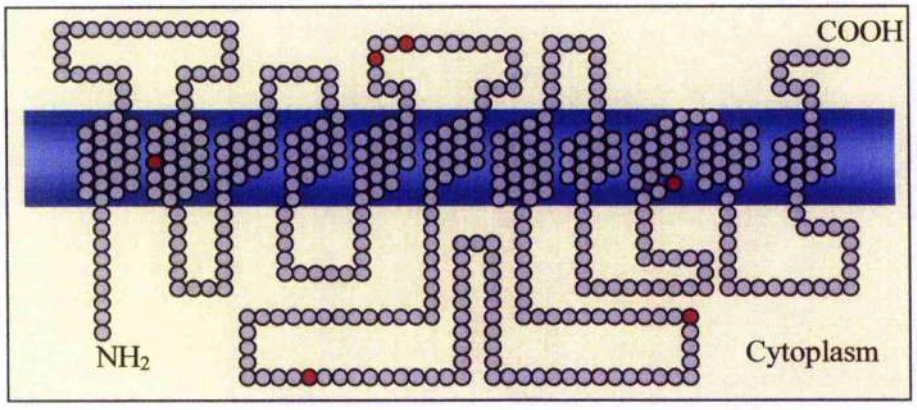


Figure 1.6 Predicted structure of TbAT1.
(R. Burchmore) Amino acid sequence changes in STIB 777R are highlighted in red. In sequence these are L71V, A178T, G181E, D239G, N286S and L380P.

Other arsenical and diamidine resistant cell lines have been shown to have alterations in their P2 transporter. A diamidine resistant strain of *T. equiperdum*, derived by sub-curative dosing *in vivo*, has been found to have a much reduced P2-like adenosine transport component.

Because of the residual P2-like activity, it seemed likely that the change in P2 activity was due to a loss in affinity for the drugs as substrates, rather than a loss in P2 function (Barrett et al. 1995).

T. b. brucei 247 mr and *T. b. gambiense* 386 mr are arsenical resistant cell lines, both of which were derived *in vivo* by serial passage in increasing sub-curative doses of cymelarsan (Scott et al. 1996). *T. b. brucei* 247 mr has recently been shown to lack an open reading frame for *TbAT1*, and *T. b. gambiense* 386 mr has an intact open reading frame, but lacks a detectable *TbAT1* transcript (R. Burchmore, unpublished data).

A *T. evansi* cell line that was selected for resistance to cymelarsan, and was also resistant to berenil treatment had lost adenosine transport via P2. The wild type parent strain is reported, however, to have three adenosine transporters, unlike other studies which report only two (Ross & Barns 1996).

A study of CSF infections of patients from a focus in north-western Uganda has also identified mutations in *TbAT1* in trypanosomal isolates from both newly infected cases and relapse patients (Matovu et al. 2001b). Their findings showed that all of the isolates that showed a mutant genotype by restriction enzyme fragment length polymorphism (RFLP) and single stranded conformational polymorphism (SSCP) had the same mutations in *TbAT1*. Out of the 9 point mutations present in these extracts, 8 are identical to those found in the laboratory derived drug resistant STIB 777R strain. Furthermore, two more isolates, STIB 871, a drug resistant *T. b. rhodesiense* isolate from south-eastern Uganda, and K003, a *T. b. gambiense* stock isolated from a melarsoprol relapse patient in northern Angola also showed identical point mutations in *TbAT1*, which were the same as those in the CSF isolates (Figure 1.7). A representative sample of CSF isolates that showed wild type RFLP and SSCP patterns were sequenced and showed wild type *TbAT1* sequence.

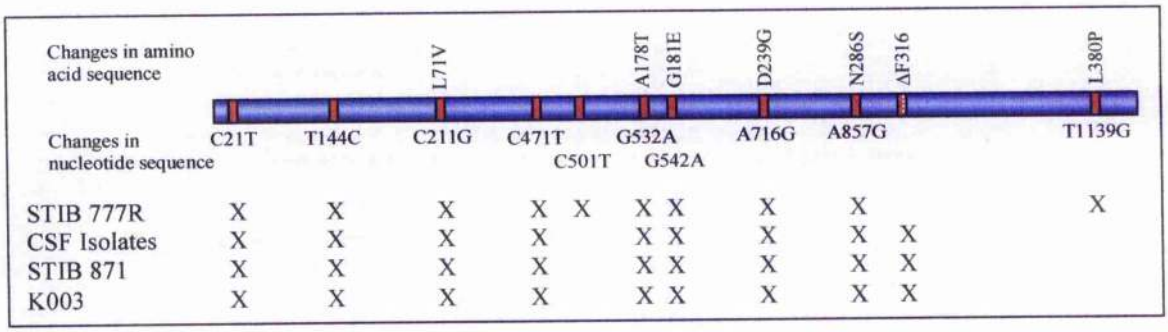


Figure 1.7 Mutations in *TbAT1* are highly conserved in drug resistant cell lines and field isolates.

From Matovu, E., 2001. The blue bar represents the *TbAT1* ORF. The mutations C21T, T144C, C211G, C471T, G532A, G542A, A716G and A857G are all present in STIB 777R, CSF isolates from Uganda, STIB 871 and K003.

One isolate, K001, a *T. b. gambiense* stock isolated from a melarsoprol relapse patient in northern Angola lacked *TbAT1* altogether, creating a 'natural knockout'. A constructed *ΔTbat1* (P2 knock out) cell line (E. Matovu, unpublished data) does not have any P2 transport activity, and shows some resistance to melarsoprol (two fold increase in IC_{50}). Many isolates from relapse patients, however, showed no difference in *TbAT1* indicating that the situation in the field regarding resistance and P2 transport is very complicated, and that other routes to resistance exist.

1.4.2 A Possible Link with Resistance to Veterinary Trypanocides

Many wild and domestic animals suffer from trypanosomiases, caused by various trypanosome species, most notably *T. congolense* and *T. vivax*, but also *T. b. brucei*, *T. equiperdum* and *T. evansi*. Infection in domestic cattle causes a disease known as Nagana, which can cause a 20 % decrease in calving, a 25 % decrease in milk production as well as an estimated 3 million livestock deaths per year (Seed 2000). Outbreaks of Nagana and HAT restrict land use and inhibit agricultural development of otherwise agreeable rural areas.

There is also evidence that domestic cattle can act as a reservoir host for *T. b. rhodesiense*, which causes the acute form of HAT. The first report of a human infective trypanosome

species isolated from an animal is that of a strain of *T. b. rhodesiense* isolated from a bushbuck (Heisch et al. 1958). More recently DNA and isoenzyme analysis of isolates from cattle have shown that up to 23 % of infected cattle in an epidemic area contain human infective trypanosomes as indicated by the above techniques (Hide 1999).

In a recent publication it was reported that a programme to restock an area in Uganda with cattle from outwith the surrounding area has directly resulted in an outbreak of *T. b. rhodesiense* sleeping sickness (Fevre et al. 2001). The authors found that 54 % of cattle imported in a 4 year period preceding the outbreak originated from sleeping sickness endemic areas. They also found that there was a significant correlation between the distance from the main market area and the spread of the epidemic. It was unlikely that human carriers of the parasite were the cause of the outbreaks as none of the human cases presented reported travelling from or into the area. This all suggests that the cattle carried human infective parasites into the area and the native *Glossina* spp, *Glossina fuscipes fuscipes*, is responsible for their spread into the human population.

The above authors conclude with the suggestion that the testing of animals and the treatment of all cattle in the programme with one curative dose of trypanocide could curtail the dispersion of HAT into new areas. While on the surface this appears to be an exceptionally good idea, further scrutiny reveals it could be a risky strategy depending on the nature of the trypanocidal agent used and the competence of its application.

The growth of resistance to a drug in a field situation can be associated with one or more factors. Among the most important of these are the compound's widespread use, prophylactic use and self-administration. These can all lead to the administration of inappropriate doses, and expose the infecting organism to sub-curative doses of drug that can, over time, lead to resistance.

None of these factors are likely to occur frequently enough through melarsoprol treatment to result in resistance. Although HAT is a very significant health problem, it is still a relatively uncommon disease when compared to, for instance, malaria, for which there is widespread drug resistance. This means that melarsoprol treatment is probably not widespread enough to

lead to resistance. The drug is not used as a prophylactic and requires hospitalisation for treatment, so the administration of sub-curative doses is rare.

The treatment of cattle with berenil to treat Nagana, however, does fit all the above criteria to raise resistance to berenil. Berenil is administered by farmers and is often used as a prophylactic. The compound should be purchased from pharmacies, but is widely available on the black market. A survey of these black market products showed that they could contain between 0 and 90 % diminazene aceturate, the active component of berenil. More worryingly, legally obtained products were also found to contain hugely varying amounts of diminazene aceturate (Tetty et al. 2002). Add to this the fact that farmers may underestimate the weight of their cattle, and may not treat an entire herd, but only those that appear ill, and the likelihood of a trypanosome encountering a sub-curative dose of berenil becomes very high.

Both berenil and melarsoprol, or their active metabolites, exploit the trypanosome specific P2 purine transporter to enter the parasite cells, and the development of drug resistance is closely related to the loss of this transporter (Barrett & Fairlamb 1999). It is relatively easy to select for cross-resistance between berenil and *in vitro* and *in vivo*, and cross-resistance has been observed in the field. Human infective trypanosomes infecting cattle could potentially develop a resistance to berenil and carry cross-resistance to melarsoprol into humans.

The importance of this pathway to melarsoprol resistance in *T. b. gambiense* infections is uncertain. This parasite has been reported to exist in domestic animals, but only to a very limited extent. It is in fact unlikely that this reservoir could play any role in the epidemiology of the disease (Noireau et al. 1986). In *T. b. rhodesiense*, however, upwards of 20 % of domestic cattle can be carrying human infective pathogens which could develop resistance to berenil treatment leading to cross resistance to melarsoprol.

1.5 Nutrient Transporters Implicated in Drug Uptake and Resistance

Several classes of nutrient tranporters have been identified in *T. brucei*. Among these are the purine, glucose and amino acid transporters, and the ongoing trypanosome genome project is likely to identify even more. As around 5-10 % of the genomes of most organisms encode

membrane transporters, it is probable that the *T. brucei* genome carries around 400 transporter genes (Borst & Fairlamb 1998).

At least 3 trypanosome transporters have been identified as being able to carry drugs into the trypanosomes, and their activity may be the reason for the selectivity of these drugs for trypanosomal cells as opposed to mammalian cells.

1.5.1 Purine Transporters

Trypanosomes are unable to synthesise the purine ring *de novo*, relying upon their external environment for a supply (Hammond & Gutteridge 1984). To facilitate this they have developed an array of salvage enzymes and purine transporters, among which are the P1 and P2 nucleoside transporters (Carter & Fairlamb 1993). Targeting trypanosome purine transporters or salvage enzymes in the design of new trypanocidal compounds is relatively futile due to the large amount of redundancy built into the system. Whilst blocking purine uptake would doubtless be detrimental to the trypanosomes, to do so would require a cocktail of inhibitors to be administered to the patient.

In comparison, mammalian cells are able to make the purine ring and therefore have less need to salvage purines from the environment (Griffith & Jarvis 1996). Most mammalian nucleoside and nucleobase transporters recognise a wide variety of substrates, including both purines and pyrimidines, and have a lower affinity for their substrates than the trypanosome transporters (de Koning & Jarvis 1999).

The P2 transporter is of particular interest being able to facilitate the internalisation of a wide range of trypanocidal drugs. Both the P1 and P2 transporters have been extensively characterised, and their recognition motifs, the part of the substrate molecule that the transporter recognises and binds to prior to internalisation, have been established.

1.5.1.1 The P1 Transporter

The P1 transporter is a very high affinity and high capacity transporter for adenosine (K_m = 0.38 +/- 0.1 μM, V_{max} = 2.8 +/- 0.4 pmol / 10^7 cells / s) and inosine, and shows a high

affinity for all natural purine nucleosides (de Koning & Jarvis 1999). As for other characterised purine transporters (de Koning & Jarvis 1997), P1 has been shown to be driven by protonmotive force (de Koning et al. 1998). The recognition motif for P1 is illustrated in Figure 1.8.

The ribose moiety is essential for binding to P1. The hydroxyl groups at positions 3 and 5 are essential, but the hydroxyl group at position 2 does not appear to be involved in binding. The nitrogens at positions 3 and 7 are also required for high affinity binding. Although C2 is not directly involved in binding to the transporter, substitutions at C2 can reduce the negative charge at N3, slightly reducing affinity. The same happens at N7 with substitutions at C8 and N9 (de Koning & Jarvis 1999).

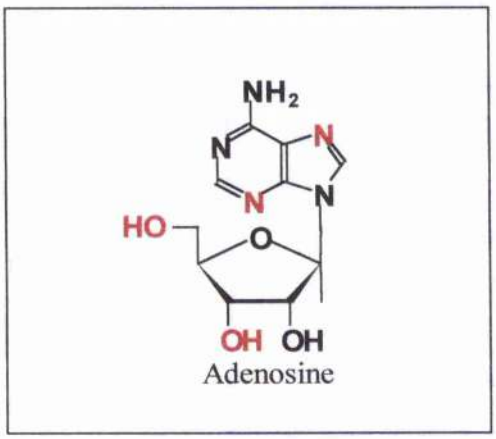


Figure 1.8 Adenosine, highlighting the P1 recognition motif.
The primary recognition motif is shown in red.

1.5.1.2 The P2 Transporter

The P2 transporter has a high affinity for 6-amino purines, its natural substrates being adenosine (with a K_m of 0.92 +/- 0.06 μM ,and a V_{max} of 1.12 +/- 0.08 pmol / 10^7 cells / s) and adenine (de Koning & Jarvis 1999). It has not been shown how transport by P2 is mediated, but it is likely that it is also protonmotive force driven. The recognition motif for P2 is illustrated in Figure 1.9.

The selectivity of P2 for purines suggests that the purine ring is important in the binding motif, and the most important groups involved in substrate recognition are the amine group at position 6 and the nitrogen at position 1 of the purine ring. These two groups together are seen as the primary P2 recognition motif: $H_2N\text{-}C(R_1)=N\text{-}R_2$. The aromaticity of the purine ring may also contribute to binding through π-stacking interactions, and substitutions at C2 reduce affinity, possibly by reducing the electron density at N1. N3 and N7 are not involved in binding.

The ribose moiety is not important for binding to P2. In fact adenine, which lacks the ribose group, has a slightly higher affinity for P2 than adenosine. Substitutions at N9 do affect binding, but only to a very small extent (de Koning & Jarvis 1999).

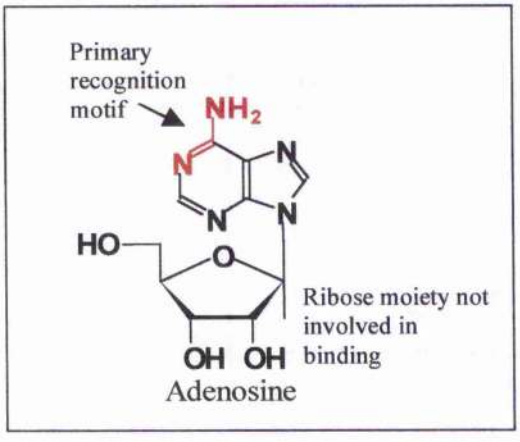


Figure 1.9 Adenosine, highlighting the P2 recognition motif.
The primary recognition motif is shown in red.

1.5.1.3 Exploitation of the P2 Transporter for Drug Uptake

One approach to the design of new trypanocides, which is used in this study, is that of targeting compounds to the parasite through a specific transporter with a unique recognition motif. P2 is a good candidate as a 'gateway' for drug targeting in this way as substrates with the P2 binding motif, $H_2N\text{-}C(R_1)=N\text{-}R_2$, show a high affinity to P2 and a low affinity for mammalian transporters. The P2 transporter also concentrates substrates within the cell, and there is a low abundance of natural substrate in the bloodstream that would compete for binding sites with any potential drugs. Toxic moieties linked to the P2 recognition motif

would hopefully be less toxic to mammals as the target cells would take up the majority of drugs. Also, due to the concentrative nature of P2, less drug would need to be delivered into the mammal, reducing the effective dose and the risk of toxicity even further. This approach has already been used as a mode of delivery for polyamine analogues (Tye et al. 1998), nitric oxide releasing compounds (Soulère et al. 2000), and organotin compounds (Susperregui et al. 1999).

P2 has been linked with drug resistance in the lab, generally through the loss of function of the transporter. If this situation were to be mirrored in the field there would be a significant risk that compounds designed in this way, to be internalised through the P2 transporter, would rapidly lose their usefulness due to resistance. The situation regarding P2 and drug resistance in field situations is not clear however. Although there are scattered reports of a connection between P2 status and drug resistance, there are also reports, often in the same studies, of drug resistance that appears to be unrelated to P2 transport (Brun et al. 2001). Any new compound is also likely to be administered by trained staff under controlled conditions, lessening the likelihood of further resistance developing.

1.5.2 Pentamidine Transport

Pentamidine is able to cross the trypanosome cell membrane using the P2 transporter. Two additional transporters for pentamidine have also been identified (de Koning 2001b). These are the high affinity, low capacity transporter (HAPT1) and the low affinity, high capacity transporter (LAPT1).

The high affinity transporter has a K_m value of 0.036 +/- 0.006 μM, and a V_{max} of 0.0044 +/- 0.0004 pmol / 10^7 cells / s. Although HAPT1 has a very high affinity for pentamidine, the transporter is quickly saturated and uptake via HAPT1 is only likely to be significant at very low extracellular concentrations (< 0.01 μM). The low affinity transporter has a K_m value of 56.2 +/- 8.3 μM, and a V_{max} of 0.85 +/- 0.15 pmol / 10^7 cells / s. This transporter is able to carry more pentamidine across the cell membrane but, due to its very low affinity for pentamidine, only becomes of significance at higher concentrations (>50 μM). The P2

transporter falls between these two in terms of K_m (0.26 +/- 0.03 μM), and V_{max} (0.068 +/- 0.007 pmol / 10^7 cells / s), and at any physiologically significant concentration of pentamidine is responsible for at least 50 % of transport into the trypanosome cell (Figure 1.10).

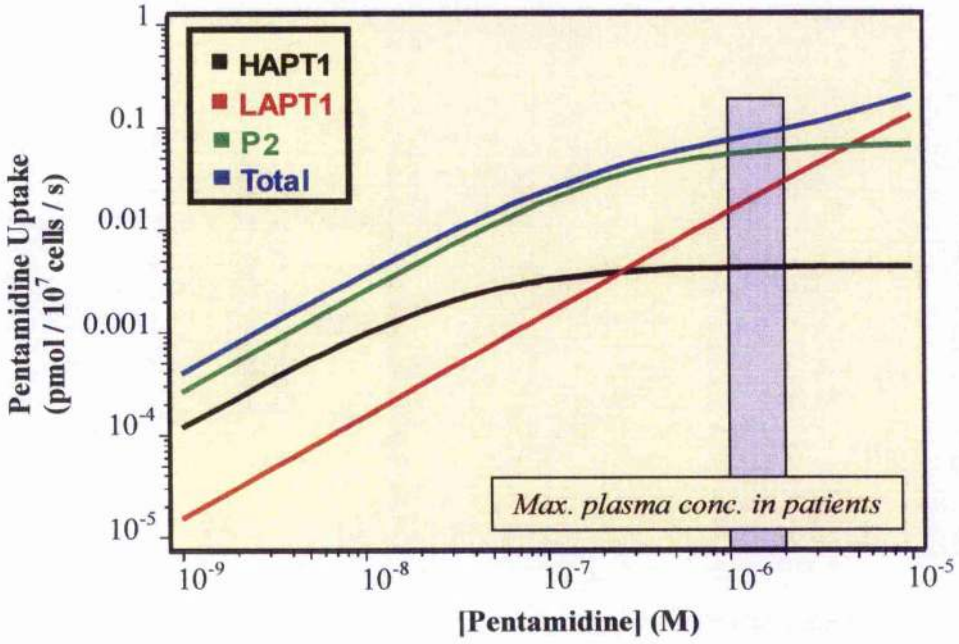


Figure 1.10 Pentamidine uptake rates by the various transporters in *T. b. brucei* bloodstream forms.

Reproduced from Bray P. G., 2003. Uptake rates are plotted as a function of the extracellular pentamidine concentration from $V = (V_{max} \times L)/(L + K_m)$ in which L is the ligand concentration. Plasma concentrations in patients treated with the standard 4 mg/kg dosing have been determined at 0.5 – 2.5 μM (Waalkes & DeVita 1970).

1.6 Potential Drug Targets

As described previously, the current drugs available for the treatment of trypanosomiasis are mostly over fifty years old, some are toxic and there is increasing resistance. The development of new chemotherapeutic compounds is of great importance, and the search focuses on differences between the biochemistry and metabolism of the parasites and their mammalian hosts that can be exploited as drug targets. The trypanosome genome project has aided in the identification of potential targets through comparative genetics, and lead compounds have been designed as inhibitors of trypanosome specific targets. Targets have been further

validated in gene knockout experiments (Clayton 1999; Kelly et al. 1995) and, more recently, using RNAi methods (LaCount & Donelson 2001).

1.6.1 Purine Transport and Metabolism

Purines are essential to trypanosomes, their uptake and metabolism is different in many ways from mammalian systems (Marr 1991), and blocking purine transporters or salvage enzymes could be sufficient to stop parasite growth. The parasite however, has many purine transporters, recognising different substrates, which can then be interconverted by different salvage enzymes. Effective blocking of purine metabolism would require the administration of a large mixture of inhibitors to patients, which is impractical. As previously described, however, many nutrient transporters, including the purine nucleoside and nucleobase transporters, could act as gateways for the transport of trypanocides specifically into trypanosomes.

1.6.2 Polyamine Metabolism

Polyamines in African trypanosomes include putrescine and spermidine, which have 2 or 3 amine groups respectively. A glutathione-spermidine conjugate called trypanothione is also unique to this group of organisms. Polyamine metabolism has been validated as a target in trypanosomes by the action of DFMO on ornithine decarboxlyase, the first enzyme in the polyamine synthesis pathway (Giffin et al. 1986; Wang 1995).

Further studies on the polyamine pathway have focused on inhibiting the action of S-adenosylmethionine decarboxylase, a crucial enzyme in the synthesis of putrescine and spermidine. The compounds CGP 40215, a diamidine produced by Ciba-Geigy as part of their anticancer programme, and methylgloxyl bis(guanylhydrazone) have been screened for their therapeutic effects. Their therapeutic activity was found to be limited to early stage infections, and the compounds were subsequently abandoned as potential drugs (Keiser et al. 2001).

1.6.3 Trypanothione Metabolism and Oxidative Stress

Trypanosomes contain trypanothione, a glutathione-spermidine conjugate that functionally replaces glutathione found in most cell types. The parasite specific enzyme, trypanothione reductase, is critical in maintaining reduced trypanothione to maintain an intracellular reducing environment (Fairlamb & Le Quesne 1997; Kreiger et al. 2000). The absence of trypanothione reductase from the cell results in a loss of virulence and leaves trypanosomes vulnerable to attack by oxidative stress (Kreiger et al. 2000). The inhibition of trypanothione reductase or blocking trypanothione production are considered as targets for drug development (Schmidt & Krauth-Siegel 2002).

Trypanosomes also lack the enzymes catalase and glutathione peroxidase, which in most eukaryotes detoxify hydrogen peroxide. The nitrofuran, Nifurtimox, has activity against *T. cruzi* and has also been suggested for use in cases of melarsoprol refractory *T. brucei* infections (Pepin et al. 1992) Its activity is mainly attributed to the reduction of the nitro group to form the nitro anion free radical ($NO_2\cdot^-$). This reactive species may then exert its toxicity by interacting with parasite macromolecules, or by reacting with oxygen to produce reduced oxygen by-products such as $O_2\cdot^-$ or H_2O_2 that could cause oxidative stress within the cell.

1.6.4 Other Potential Drug Targets

1.6.4.1 Glycolysis

Trypanosomes rely upon glucose metabolism for all their energy production. Many enzymes of glycolysis in trypanosomes are functionally and structurally different from mammalian enzymes, partly due to the fact that the first seven enzymes of the pathway reside within unusual peroxisome-type organelles called glycosomes (Michels et al. 2000.). Inhibitors of the glycolytic enzymes are being pursued (Verlinde et al. 2001).

1.6.4.2 The Pentose Phosphate Pathway

In trypanosomes, enzymes of the pentose phosphate pathway (Barrett 1997; Cronin et al. 1989), as well as other pathways, are related to cyanobacteria rather than to those of

eukaryotes (Hannaert et al. 2003). The third enzyme of the pentose phosphate pathway, 6-phosphogluconate dehydrogenase, is essential to trypanosomes, and selective inhibitors have been designed to exploit the structural differences of the trypanosome enzyme relative to its mammalian counterpart (Barrett & Gilbert 2002).

1.6.4.3 Kinetoplast/RNA Editing

The kinetoplast is the unusual mitochondrial DNA network that exists in Trypanosomes (section 1.2.3). It has a very complex topology maintained by topoisomerases, which are responsible for maintaining this topology throughout replication, transcription and recombination. These topoisomerases may provide targets for drug design (Wang 1994).

RNA editing is a process unique to trypanosomes. In this respect, components of this process may provide a promising target for drug development (Stuart et al. 1997).

1.6.4.4 Lipid and Sterol Metabolism

Ergosterol is the major sterol in trypanosomatids, whereas in mammals, the predominant sterol is cholesterol. The enzymes involved in sterol biosynthesis in trypanosomes vary accordingly, and act as potential targets. Inhibitors of $\Delta^{24(25)}$ sterol methyl transferase inhibit the growth of the related trypanosomatids, *Crithidia fasciculata* (Rahman & Pascal 1990) and *T. cruzi* (Urbina et al. 1996).

1.7 The Role of the P2 Purine Transporter in Drug Uptake and Resistance

Any potential drug designed to affect any of the parasite specific targets described above will have to cross the trypanosome cell membrane. A lipophilic compound that can cross any cell membrane, including that of the mammalian host, could achieve this. However, although toxic or inhibitory compounds can be designed to a target that is specific to the parasite cell, a compound that has access to any cell increases the risk of toxicity to the host cell.

Another strategy for crossing the trypanosome cell membrane is to exploit the action of a parasite membrane transporter with a high affinity for a particular substrate, and a recognition

motif for that substrate unlike any in mammalian cells. By using this transporter as a gateway, it should be possible to target compounds to the parasite cell specifically. The P2 aminopurine transporter fulfils this requirement as a 'gateway' for parasite specific uptake in that it requires a recognition motif which appears to be very unusual and for which it has a high affinity (Hasne & Barrett 2000). With this objective in mind, compounds were designed and tested for their ability to interact with the P2 transporter and their overall toxicity to trypanosomes *in vitro* and, where appropriate, *in vivo*.

The P2 transporter has other properties that lend themselves to its being used as a gateway. The natural substrates for this transporter, adenine and adenosine, are found at very low concentrations in the bloodstream. This means they will offer very little competition for the transporter. Also, the P2 transporter is likely to be driven by a protonmotive force, and actively accumulates substrate against the concentration gradient. If it were able to do this with a trypanocide, less compound would have to be administered to the patient and yet achieve high concentrations within the parasite, reducing the likelihood of toxic side effects.

In spite of its potential utility as a novel gateway, the loss of the P2 transporter has been linked with drug resistance in laboratory strains, and has been reported to be involved in some cases of drug resistance in the field. Because of fears about the development of drug resistance, this study also seeks to further clarify the molecular basis of drug resistance and the role of the P2 transporter. To this end, the molecular basis for the development of drug resistance in a *T. equiperdum* strain that has been reported to have a P2 transport component with a greatly reduced affinity for adenosine has been investigated.

A test for drug resistance through the loss of an active P2 transporter is also pursued. Tests related to P2 transport have been suggested previously, however, these are unsuitable for use in the field, where they would be most useful, to both clinicians and scientists investigating the phenomenon of drug resistance in African trypanosomes.

Chapter II

Materials and Methods

2.1 Trypanosome Cell Lines

2.1.1 *Trypanosoma brucei brucei* 427

T. b. brucei strain EATRO (East African Trypanosomiasis Research Organisation) 427 was used routinely for uptake assays. The strain is reported to have been isolated from a sheep in Uganda (Cunningham & Vickerman 1962) and is extensively used for experimental purposes as very high parasitaemias can be achieved in experimental animals. Most biochemical analyses in *T. brucei* have been done using this strain. These stocks were supplied by Dr. M. P. Barrett, University of Glasgow.

2.1.2 *T. b. brucei* 427, MiTat 1.2 (BS221) and *ΔTbat1* mutant clone

The *ΔTbat1* (P2) null mutant clone was constructed by sequential replacement of *TbAT1* with the neomycin and puromycin resistance markers in *T. b. brucei* 427, MiTat 1.2 (BS221). The knockout cell line was 4-fold less sensitive to melarsoprol, melarsen oxide and 18.6-fold less sensitive to diminazene aceturate (Enock Matovu, unpublished data). These stocks were originally supplied by Enock Matovu and are held at the University of Glasgow.

2.1.3 *T. b. brucei* 247 and 247 mr

STIB (Swiss Tropical Institute, Basel) 247 was originally isolated from a hartebeest in 1971 in the Serengeti National Park, Tanzania (Jenni et al. 1986). The cymelarsan resistant 247 mr cell line was derived by serial passage in mice treated with subcurative doses of cymelarsan. The resulting clone was found to be 130-fold less sensitive to cymelarsan and 16-fold less sensitive to berenil *in vivo* (Scott et al. 1996). These stocks were originally supplied by Dr. C. M. R. Turner, University of Glasgow. The *T. b. brucei* 247 mr cell line has been shown to lack the *TbAT1* open reading frame, resulting in a 'natural knockout' of P2 transport function (R. Burchmore, unpublished data).

2.1.4 *T. b. gambiense* 386 and 386 mr

STIB 386 was originally isolated from a man in Daloa in the Ivory Coast. The cymelarsan resistant 386 mr cell line was also derived by serial passage in mice treated with subcurative doses of cymelarsan. *T. b. gambiense* strain 386 mr is 20-fold less sensitive to cymelarsan *in vivo* (Scott et al. 1996). These stocks were originally supplied by Dr. C. M. R. Turner, University of Glasgow. 386 mr carries a full open reading frame for *TbAT1*, but lacks any detectable transcript of that gene (R. Burchmore, unpublished data).

2.1.5 *T. equiperdum* BoTat 1 P and PBR

T. equiperdum BoTat 1 (Bordeaux trypanozoon antigenic type 1) P originated from a stock held at the Pasteur Institute, Paris. The berenil resistant line, *T. equiperdum* PBR was derived from the parent cell line by serial passage through mice treated with sub-curative doses of berenil (Zhang et al. 1993). The resistant cell line is 35.6-fold less sensitive to berenil and 4-fold less sensitive to cymelarsan *in vitro*. *In vivo*, the resistant cell line is insensitive to berenil up to the maximum tolerated dose in Swiss mice, and 4-fold less sensitive to cymelarsan. These stocks were originally supplied by Dr. Theo Baltz, Université de Bordeaux, and are held at the University of Glasgow.

2.2 Growth and Maintenance of Trypanosome Stocks

2.2.1 Bloodstream Form Trypanosomes

2.2.1.1. *In vivo* culture

Trypanosomes were stored in capillary tubes, in liquid nitrogen storage facilities, in blood containing 10.5% DMSO. Wistar rats or ICR mice (depending on the number of trypanosomes required) were infected by intraperitoneal injection of the parasite stabilates. Parasitaemias were checked daily by examination of a tail blood smear under phase contrast. Parasitaemia was scored using the rapid matching method (Herbert & Lumsden 1976).

When the parasitaemia reached $1x10^9$ cells/ml the animal was sacrificed using CO_2 and the blood collected by cardiac puncture in a syringe containing 100 U/ml heparin in CBSS (Appendix I).

2.2.1.2 *In vitro* culture

Cultures were incubated in sterile culture flasks at 37°C and 5% CO_2 in HMI-9 medium (Hirumi & Hirumi 1989) containing 20% heat-inactivated foetal calf serum. 5 ml cultures were seeded with 200 µl of culture at 2×10^6 cells/ml. Cells were allowed to grow for 3 days after which they had reached maximum density (approximately 2×10^6 cells/ml) and were passaged again in the same way.

2.2.2 *In vitro* Culture of Procyclic Form Trypanosomes

Cultures were incubated in sterile culture flasks at 28°C and 5% CO_2 in SDM-79 medium (Brun & Jenni 1977) containing 10% heat-inactivated foetal calf serum. 5 ml cultures were seeded with 200 µl of culture at 10^7 cells/ml. Cells were allowed to grow for 3 days after which they were passaged again in the same way.

2.3 Chemicals

2,5-bis (4-amidinophenyl) furan (DB 75) and 2,5-bis (4-amidinophenyl) 2,3-bis methylfuran (DB 544) were gifts from Dr R. Tidwell, University of North Carolina and Prof. D. Boykin, Georgia State University. Polyamine analogues, HIV gp120 binding mimetics and the nitrofuran and nitroimidazole compounds were synthesised in Cardiff at the Welsh School of Pharmacy by Burkhard Klenke, Gorka Jimenez-Bueno, C. Boussard and Ian Gilbert. Alamar Blue was obtained from Trek Diagnostic System Ltd. Oligonucleotides were synthesised by MWG Biotech. Restriction enzymes and *Pfu* DNA polymerase were purchased from Promega. Radiolabeled [2,8, -^{3}H] adenosine (35.9 Ci/mmol) was purchased from Perkin Elmer, [ring -^{3}H] Diminazene aceturate (83 Ci/mmol) was purchased from Amersham Pharmacia Biotech (custom synthesis) and [α^{32}P] ATP (3000 Ci/mmol) was purchased from NEN Life Science products. All other chemicals were of the highest grade available from Sigma.

2.4 Transport Assays

2.4.1 Separation of Trypanosomes from Blood

Parasites were separated from blood using a DEAE cellulose column (Lanham & Godfrey 1970). Whole blood containing the parasites was centrifuged at 1250 x g for 15 minutes. The trypanosomes form a layer over the red blood cell pellet, which was removed by pipetting. The trypanosome layer was then resuspended in separation buffer, PSG (Appendix I), and applied to the DEAE cellulose (DE-52) column, where under standard conditions, the red blood cells are negatively charged, and adhere to the cellulose, and the trypanosomes are positively charged and can pass through the column to be collected beneath.

Trypanosomes were further prepared for use in uptake assays by washing three times in ice cold CBSS to remove soluble blood components. After the final wash, cells were resuspended to 2 x 10^8 cells/ml in CBSS in preparation for the assay.

2.4.2 Uptake Assays

Uptake assays were based on a protocol described by Carter and Fairlamb, 1993 (Carter & Fairlamb 1993).

2.4.2.1 Preliminary Uptake Assay – Establishing the Required Range

The approximate concentration that inhibited 50% of radiolabeled adenosine uptake in trypanosomes (IC_{50}) was established in order to select the range of concentrations of inhibitor to use in the full uptake assays.

100 µl of CBSS containing 0.05 µM radiolabeled adenosine and 1 mM inosine (to block adenosine uptake via the P1 transporter) was layered over 100 µl of oil (1-Bromododecane, 97%, density 1.04 g/ml). Experiments included four 10-fold dilutions, from 1000 µM, of the potential inhibitor added to individual tubes, and a no inhibitor control.

100 µl of trypanosomes at 2 x 10^8 cells/ml in CBSS, purified from blood as detailed above, were added to the experiment tubes and incubated for 10 s at 25°C. After incubation the reaction tubes were centrifuged at 10,000 x g for 1 minute in a Biofuge pico benchtop centrifuge. The cells descended through the oil layer to form a pellet separated from the excess radioactivity in the aqueous phase. The entire reaction tube was flash frozen in liquid nitrogen and the oil layer containing the pellet was cut off into a scintillation vial.

2% SDS and 2.5 ml of Ecoscint scintillation fluid was added to the samples and these were left overnight to ensure against luminescence quenching. The radioactivity present was measured in a Wallac Trilux 1450 Microbeta liquid scintillation counter. The apparent IC_{50} value for the inhibitor was calculated by analysing the data with the IC_{50} value algorithm of the Grafit 4.0 software. Each experiment was done in duplicate.

2.4.2.2 K_i Determination Assay

100 µl of CBSS buffer containing 1 mM inosine (to block adenosine uptake via the P1 transporter) and either 5, 2.5, 1.25, 0.625 or 0.3125 µM radiolabeled adenosine was layered over 100 µl of oil (1-Bromododecane, 97%). Three concentrations of inhibitor compound, spanning the calculated IC_{50} value and one no inhibitor control, were added to the above tubes giving four separate dilution series.

100 µl of trypanosomes at 2 x 10^8 cells/ml, purified from blood as described above, were added to the tubes which were incubated for 10 s at 25°C. The cells were then centrifuged through the oil layer to form a pellet separated from the excess radioactivity in the aqueous phase. The entire reaction tube was flash frozen in liquid nitrogen and the oil layer containing the pellet was cut off into a scintillation vial. The radioactivity present was measured by scintillation counter. The apparent K_i value for the inhibitor was calculated using the competitive inhibition equation from the Grafit 4.0 software. Each experiment was done in duplicate.

2.4.2.3 Inhibition of Uptake of radiolabeled Diminazene Aceturate and Adenosine by an Excess of Transport Substrates

The uptake of 20 μM Diminazene Aceturate (berenil) and 10 μM adenosine was measured over 10 minutes at 25°C.

1 mM concentrations of adenine, inosine and hypoxanthine, either singly or together, were combined in 100 μl of CBSS containing radiolabeled substrate. 100 μl of trypanosomes at 2 x 10^8 cells/ml, purified from blood as described above, were added to the tubes, which were then incubated for either 30, 60, 120, 300 or 600 s at 25°C. The cells were then spun through the oil layer to form a pellet separated from the excess radioactivity in the aqueous phase. The entire reaction tube was flash frozen in liquid nitrogen and the pellet cut off into a scintillation vial. The radioactivity present was measured by scintillation counter. Each experiment was conducted in triplicate.

2.5 Trypanotoxicity

2.5.1 *In vitro* Toxicity Assay (Alamar Blue Assay)

The Alamar Blue Assay (Raz et al. 1997) was used to assess toxicity *in vitro*. Doubling serial dilutions of test compound were set out in a 96 well plate in duplicate, in a volume of 100 μl HMI-9. 100 μl of trypanosomes at 2 x 10^5 cells/ml where added to each well. After 48 h incubation at 37°C and 5% CO_2, 10% Alamar Blue (20 μl) was added to each well and the plates incubated for a further 24 h. Unspecified enzymes in live cells reduce the Alamar Blue to a pink colour. The concomitant change in absorption at 600 nm can be measured spectrophotometrically, or fluorometric measurements can be taken at 530 nm excitation and 590 nm emission as the amount of fluorescence detectable at these wavelengths increases in the reduced dye. All measurements were taken fluorometrically. The IC_{50} value was calculated by analysing the data with the IC_{50} value algorithm of the Grafit 4.0 software. Values were checked for accuracy by visually examining cell viability and motility. Each experiment was performed in duplicate, and replicated at least once.

2.5.2 *In vivo* Toxicity Assay

Groups of five female ICR mice were infected intraperitonealy with 2 x 10^5 parasites. Each group was treated on days 2 and 3 post infection with either test compounds at the highest tolerated dose, berenil at 20 mg/kg, or no drug. Parasitaemias were checked daily by examination of a tail blood smear under phase contrast microscopy. Parasitaemia was scored using the rapid matching method (Herbert & Lumsden 1976). Any animal approaching 1 x 10^9 trypanosomes/ml was sacrificed. Animals presenting no parasitaemia were allowed to continue in the experiment until 40 days post infection.

2.6 Fluorescence Assays

Blood was collected as previously described from ICR mice presenting peak parasitaemias. The parasitaemia was variable according to the infecting trypanosome strain. 0.5 µl of 10 mM fluorescent diamidine (DB 75, DB 544 or DAPI) was added to 500 µl of whole blood at room temperature to give a starting concentration of 10 µM. This was thoroughly mixed and approximately 2 µl removed to a glass slide and covered to produce a wet blood smear that was viewed over a 10-30 minute time period. This was viewed directly through the Zeiss Axioscop FL fluorescence microscope using a Zeiss 02 filter at an excitation wavelength of 330 nm and an emission wavelength of 400 nm. Images were obtained by the same method, but using an Axiovert 200M Fluorescence microscope.

2.6.1 Blocking the development of fluorescence

The development of fluorescence was blocked by pre-incubating 500 µl of whole blood containing parasites with 1 mM adenosine or pentamidine for 30 seconds. The assay was then continued as described above.

2.7 Molecular Techniques

2.7.1 Purification of DNA and RNA from Trypanosomes

Bloodstream form trypanosomes were harvested from culture. RNA was extracted using TRIzol® Reagent (Life Technologies) using the manufacturer's protocol.

DNA was extracted from a cell pellet to which was added 500 µl of lysis buffer (Appendix I). This was incubated overnight at 50°C. 500 µl phenol was added and incubated for 5 minutes at room temperature, then 500 µl of chloroform added. After mixing, the tubes were centrifuged at 1800 x g for 10 minutes, and the aqueous phase removed and added to 0.1 volume 3M NaCl, 2 volumes ethanol in a 1.5 ml eppendorf. The precipitated DNA was removed and washed in 1 ml 70% ethanol. The DNA was pelleted and most of the ethanol drained off. Excess ethanol was then evaporated overnight at 37°C. The DNA pellet was resuspended in 50 µl double distilled, autoclaved water.

2.7.2 Polymerase Chain Reactions

50 µl PCR reactions were performed to amplify the *TbAT1* gene. Each reaction contained 5 µl 10x reaction buffer (provided by the manufacturer), 200 µM each of dATP, dCTP, dGTP and dTTP, 2.5 µM each of the primers, 1.25 units of *Pfu* Polymerase and 200-300 ng of DNA template. The primers for *TbAT1* amplification were designed to fall outwith the open reading frame for P2, and were,

5' P2F : CAT GCG CTT TGG TGG AGG
3' P2R : TTG GCG AAT CGG TGT ACG

Amplification was performed on a Gene AmpPCR System 2400 from Perkin Elmer under the following conditions: 5 minutes at 94°C (initial denaturation step), followed by 30 cycles of 1 minute at 94°C (denaturation step), 1 minute at 60°C (annealing step) and 2.5 minutes at 72°C (amplification step). A final amplification step was added of 72°C for seven minutes.

2.7.3 Agarose Gel Electrophoresis of DNA

Agarose gels were prepared in TAE buffer (Appendix I) with 1% agarose and 0.5 μg/ml ethidium bromide. Gels were run in TAE buffer either at 20 mV overnight (Southern) or at 80-100 mV for 1-2 hours. DNA in the gels was visualised by ultraviolet light in a trans-illuminator (UVP Laboratory Products).

2.7.4 Purification of DNA from Agarose Gels

DNA samples were run on an agarose gel alongside an appropriate DNA marker. DNA fragments that appeared of the right size were excised using a clean scalpel and transferred to a sterile 1.5 ml Eppendorf. The DNA was then extracted using a Qiagen gel extraction kit using the manufacturer's instructions.

2.7.5 A-tailing *Pfu* polymerase PCR Product

The pGEM-T (Promega) plasmid, which was used for all cloning, requires the addition of a single deoxyadenosine at the 3' end of the insert, however, *Pfu* polymerase produces a blunt ended product. *Pfu* PCR product was A-tailed by combining 7 μl of product with 1 μl of 10x *Taq* polymerase PCR buffer with $MgCl_2$, 200 μM dATP, 5 units of Taq polymerase and dd water to a final volume of 10 μl. The reaction was allowed to proceed for 30 minutes at 72°C.

2.7.6 Ligation into Plasmid Vector

pGEM-T was purchased ready to use. Ligation reactions were carried out at a 3:1 ratio of molar ends of insert to vector, according to the manufacturer's instructions and incubated overnight at 4°C.

2.7.7 Transformation of Competent Cells

JM109 competent cells of high efficiency were purchased from Promega. These were stored at -80°C and heat shocked according to the manufacturer's instructions. After heat shocking,

900 µl of LB medium (Appendix I) was added to the cells, which were then incubated at 37°C for one hour. The cells were then pelleted and resuspended in 100 µl of LB, then plated out on LB agar plates containing 50 µg/ml ampicillin, 1 mM isopropylthio-β-D-galactoside (IPTG) and 50 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). The plates were incubated overnight at 37°C.

2.7.8 Plasmid DNA Preparation

Single colonies were picked from LB agar plates, transferred to 5 ml of LB medium containing 100 µg/ml ampicillin and incubated overnight at 37°C with vigorous shaking. The culture was then centrifuged for 10 minutes at 1250 x g and DNA extracted from the pellet using a Qiagen Miniprep kit according to the manufacturer's instructions.

Plasmid DNA preparations were sent to MWG Biotech for sequencing. Primers internal to the *TbATI* open reading frame (ORF) (designed by MWG Biotech) were used to ensure complete double stranded coverage of the whole gene.

2.7.9 Restriction digest of DNA

Plasmid DNA was checked for inserts by restriction digest. Digestions were carried out at 37°C, with 5 units each of restriction enzyme (*PstI* and *EcoRI*), 1 µl of 10x buffer appropriate for each enzyme (provided by the manufacturer), 50-100 ng of miniprep DNA and double distilled water up to 10 µl. DNA samples were mixed with blue sample loading buffer (Appendix 1), and then loaded and run on an agarose gel alongside an appropriate DNA marker to identify fragments of the appropriate size.

Genomic DNA was digested in 100 µl volumes using 20 units of restriction enzyme and 10 µl of 10x buffer. Digests were carried out at 37°C for at least 4 hours to achieve complete digestion.

The Digest product was precipitated by adding 10 µl 3M NaCl, and 200 µl ethanol. This was incubated at -80°C for 30 minutes, and then centrifuged at 10,000 x g for 15 minutes. The pelletted DNA was washed in 500 µl 70% ethanol and centrifuged again at 10,000 x g for 15 minutes. Excess ethanol was removed by pipetting and the pellet dried for 20 minutes in a Savant DNA 100 Speedvac. The DNA pellet was resuspended in 20 µl double distilled, autoclaved water.

2.7.10 Southern Hybridization

Approximately 3 µg of genomic DNA was completely digested with restriction enzymes and separated on a 1% agarose gel. The gel was then placed in 0.25 M HCl for 30 minutes to nick the large molecular weight fragments of DNA. After washing in double distilled water the gel was placed in 0.5 M NaOH, 1.5 M NaCl for 30 minutes to denature the DNA. Finally, after again washing in double distilled water, the gel was placed in a neutralisation solution of 0.5 M Tris, pH 7.4, 1.5 M NaCl for 30 minutes. All these incubations took place at room temperature with gentle shaking.

DNA was then transferred to nylon membrane (Hybond N, Amersham) as described by Sambrook *et al* (Sambrook et al. 1989) using 20x SSC (Appendix I). DNA was fixed to the membrane using an ultraviolet cross-linking procedure (Spectrolinker XL-1000 UV linker, Spectronics Corporation), for optimal cross-linking.

The membrane was pre-hybridized with a hybridization solution containing 50% formamide, 5x SSC, 10x Denhardt's solution (Appendix I), 0.1% SDS, 20 mM NaH_2PO_4, 5 mM EDTA and 0.2 mg/ml of denatured herring sperm DNA, for 2 hours at 42°C. The radioactive probe to the full *TbAT1* open reading frame (as described in section 2.7.13) was then added and the hybridization left to continue at 42°C overnight.

The blot was then washed at 60°C for 3 x 30 minutes in wash buffer, consisting of 0.1x SSC and 0.1% SDS. After washing, the membrane was wrapped in cling-film and exposed to X-ray film (Konica Medical Film) at -70°C for varying lengths of time. Autoradiographs were

developed using a film processor (X-Ograph imaging system Compact X4) according to the manufacturer's instructions.

2.7.11 Formaldehyde Gel Electrophoresis of RNA

Formaldehyde gels were made by mixing 9.74 ml formaldehyde, 3 ml 20x Northern gel buffer (Appendix I) and 17.3 ml double distilled water to 30 ml melted 2% agarose solution. The gel mix was allowed to solidify in a casting block and, once loaded, run in 1x Northern gel buffer consisting of 25 ml 20x northern gel buffer, 45 ml formaldehyde and 430 ml double distilled water.

RNA samples were prepared by adding 1 µl of 20x Northern gel buffer, 3.5 µl of formaldehyde, 10 µl of formamide and double distilled water to a final volume of 20 µl. The samples were heated to 55°C for 15 minutes then chilled on ice. 2 µl of RNAse free loading buffer (Appendix 1) was added and the samples loaded onto the gel. The gel was run overnight in a fume hood at 25 V.

2.7.12 Northern Hybridization

Approximately 4 µg of RNA was electrophoresed under denaturing conditions on a formaldehyde gel. The gel was then directly blotted onto a nylon membrane and treated as described for the Southern Hybridization in section 2.7.10, above.

2.7.13 Labelling of DNA Probes

Approximately 200 ng of a gel purified, cloned insert of the *TbAT1* open reading frame was labelled with P^{32} using the Stratagene Prime-It II random primer labelling kit, according to manufacturer's instructions. The labelled probe was then purified using a spin column (Microspin TM S 200HR, Amersham Pharmacia Biotech) using the manufacturer's instructions. Total recovered labelled DNA was then heated to 95°C for 5 minutes to denature, and added to the hybridization buffer, regardless of membrane size.

2.8 Software

Biochemical kinetic data was handled using Grafit 4.0 (Erithracus Software). This included the determination of kinetic inhibition constants and IC_{50} values.

DNA and protein sequences were handled using the Vector NTI suite 6 package (Informax). This package includes ContigExpress for creating continuous sequences from overlapping contigs, Vector NTI for handling the DNA and protein sequences and AlignX for aligning nucleotide and protein sequences.

Chapter III

Polyamine Analogues Bearing the P2 Recognition Motif as Targeted Trypanocides

3.1 Introduction

Polyamines are essential for normal growth, maintenance and development of all cells, regardless of their origin. Any interference with polyamines, by blocking their production or functions, reduces cell viability, eventually leading to cell death. Targeting polyamine synthesis in trypanosomes additionally blocks the production of the parasite specific enzyme trypanothione. The absence of trypanothione from the cell leaves the trypanosome vulnerable to attack from oxidative stress (Krieger et al. 2000).

Trypanosome polyamine metabolism has already been validated as a drug target by the chemotherapeutic activity of the compound DL-α-difluoromethylornithine (DFMO) against infections of *T. b. gambiense*. DFMO disrupts polyamine synthesis by acting as an irreversible inhibitor of ornithine decarboxylase, blocking the polyamine synthesis pathway at the point of putrescine synthesis (Giffin et al. 1986) (Figure 3.1).

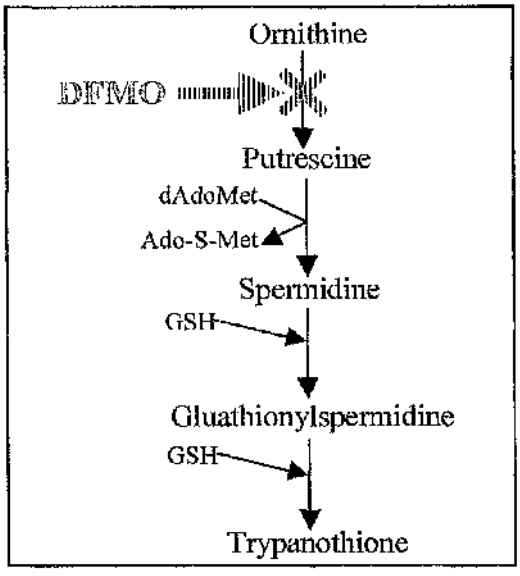


Figure 3.1. Polyamine biosynthetic pathway in African trypanosomes.
Reproduced from Fairlamb, A. H., 1997.

By introducing polyamine analogues into a cell it may be possible to compete with that cell's indigenous polyamines and disrupt their synthesis and/or function(s) within the cell. African

trypanosomes however, apparently lack any type of transporter for polyamines, all their polyamine requirements being met by *de novo* synthesis (Fairlamb & Le Quesne 1997).

The P2 transporter is normally responsible for the uptake of adenosine and adenine but has also been shown to mediate the uptake of various trypanocidal compounds (Carter et al. 1995). All compounds taken up by P2 contain a molecular motif that is recognised by the transporter and to which the transporter associates prior to uptake (de Koning & Jarvis 1999). Figure 3.2 illustrates the P2 recognition motif, $H_2N–C(R_1)=N(R_2)$, on adenosine and various trypanocides.

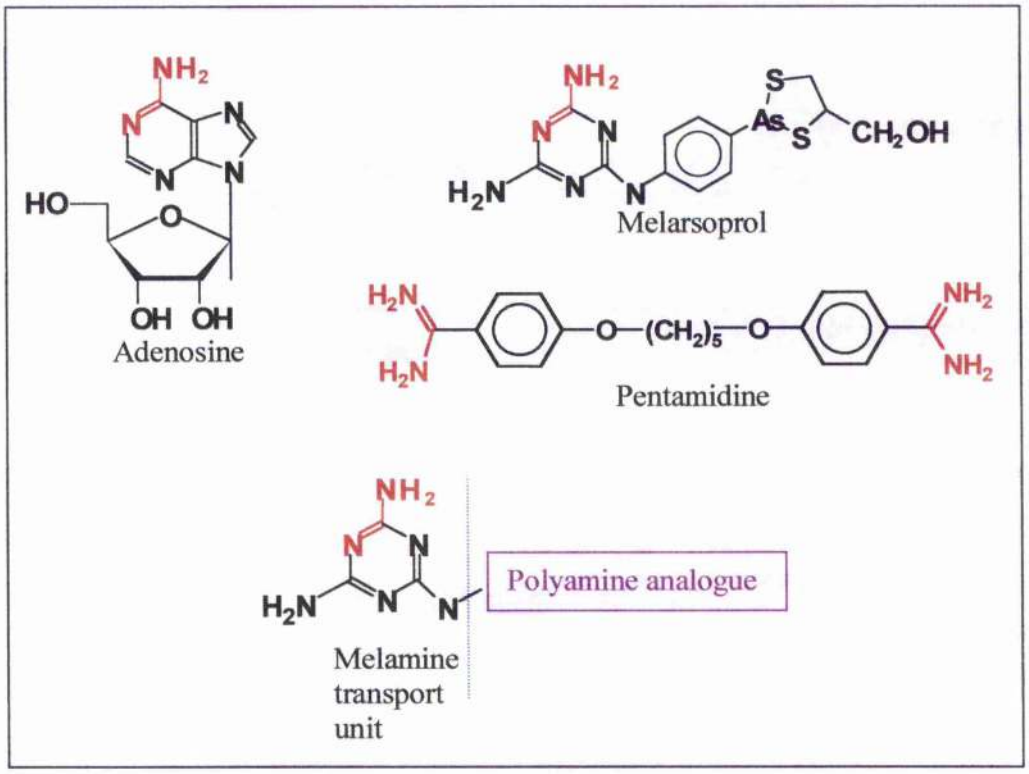


Figure 3.2. Adenosine, pentamidine, melarsoprol and melamine 'transport unit'.
The primary P2 recognition motif, $H_2N–C(R_1)=N(R_2)$, is shown in red.

In order to introduce selective uptake of polyamine analogues in trypanosomatids, a recognition motif for the P2 transporter, in the form of a melamine ring attached via a methylene linker, was added to a variety of polyamine analogues, in the hope it would act as a 'transport unit' (Figure 3.2).

Preliminary work in this area was carried out by Tye and coworkers, on a limited series of polyamine analogues. These analogues were linked to the P2 recognition motif in the form of derivatives of one or more triazine rings. The published results showed that these compounds at least associated with the P2 transporter to limit adenosine uptake, and that some showed limited toxicity to *T. b. brucei in vitro* (Tye et al. 1998).

3.1.1 Synthesis of Polyamine Analogues

Burkhard Klenke synthesised all compounds at the Welsh School of Pharmacy, Cardiff University. Each compound consisted of a polyamine analogue core unit attached to various differently substituted 1,3,5-triazines as transport units aimed at interacting with the P2 transporter (Fig. 3.3).

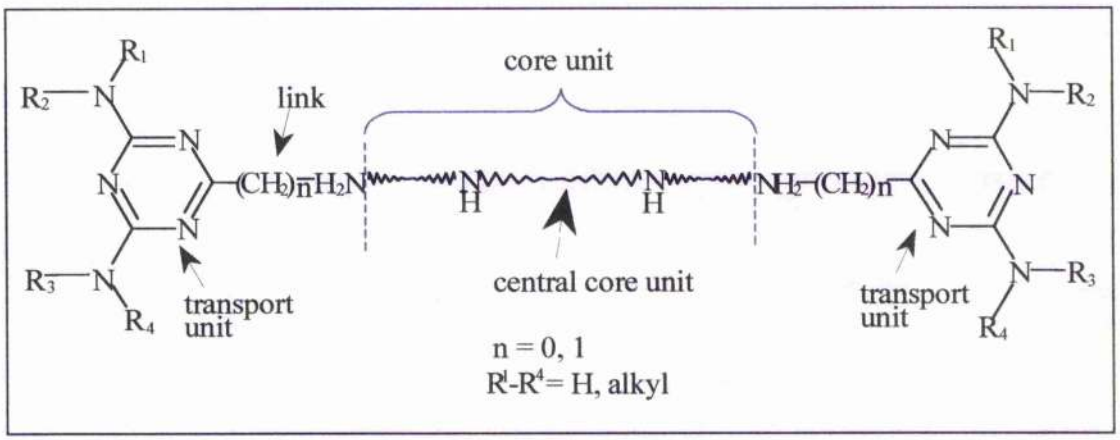
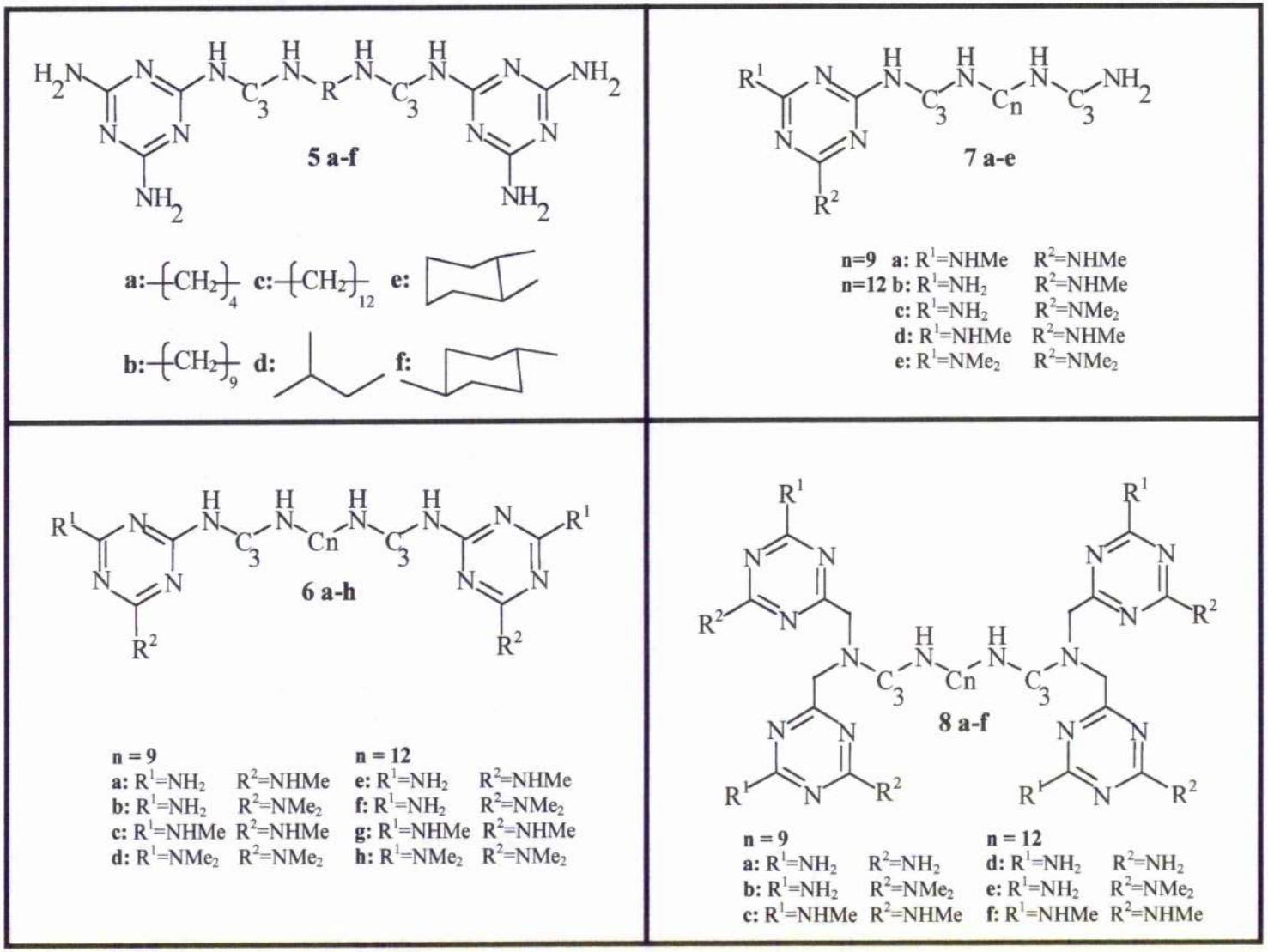


Figure 3.3. General structure of experimental compounds.

Four groups of compounds, which could be distinguished by variations of their core unit and/or transport units, were synthesised. Their structures are shown in Figure 3.4.

Figure 3.4. Structure of synthesised compounds.



3.2 Results

3.2.1 Interaction with the P2 Transporter

All compounds showed dose-dependent inhibition of adenosine uptake via the P2 transporter (Table 3.1). Apparent K_i values were calculated using the competitive inhibition equation from the Grafit 4.0 software. Examples are shown in Figure 3.5.

Compound	P2 affinity Ki (μM)	Toxicity *in vitro* IC_{50} (μM)
5a	72 +/- 5	>100
5b	19 +/- 0.6	>100
5c	0.5 +/- 0.2	>100
5d	148 +/- 12	>100
5e	55 +/- 1.14	>100
5f	125 +/- 38	>100
6a	0.75 +/- 0.25	50.4 +/- 8.2
6b	1.0 +/- 0.02	39.6 +/- 1.9
6c	14 +/- 1.67	3.9 +/- 0.7
6d	28.8 +/- 0.94	25.2 +/- 8
6e	0.135 +/- 0.004	2.0 +/- 1.5
6f	0.25 +/- 0.05	>100
6g	3.2 +/- 1.4	17.4 +/- 4.4
6h	2.5 +/- 0.2	1.67 +/- 0.5
7a	14 +/- 2	68 +/- 24
7b	0.5 +/- 0.12	>200
7c	1.1 +/- 0.1	19 +/- 2.9
7d	26.2 +/- 0.37	21.3 +/- 17
7e	0.6 +/- 0.3	14.7 +/- 1.86
8a	82.8 +/- 10.4	35.5 +/- 3.9
8b	16.78 +/- 0.11	3 +/- 0.32
8c	30.2 +/- 0.3	0.11 +/- 0.3
8d	6.7 +/- 0.5	21.2 +/- 10.1
8e	4.55 +/- 2.5	2.0 +/- 1.5
8f	2.37 +/- 0.42	2.9 +/- 0.29
Putrescine	n/a	60 +/- 11.2
Spermine	n/a	5.51 +/- 0.08
Spermidine	n/a	10.44 +/- 0.1

Table 3.1. Inhibition of adenosine uptake and toxicity of polyamine analogues.
Results are shown +/- standard error, n = 2.

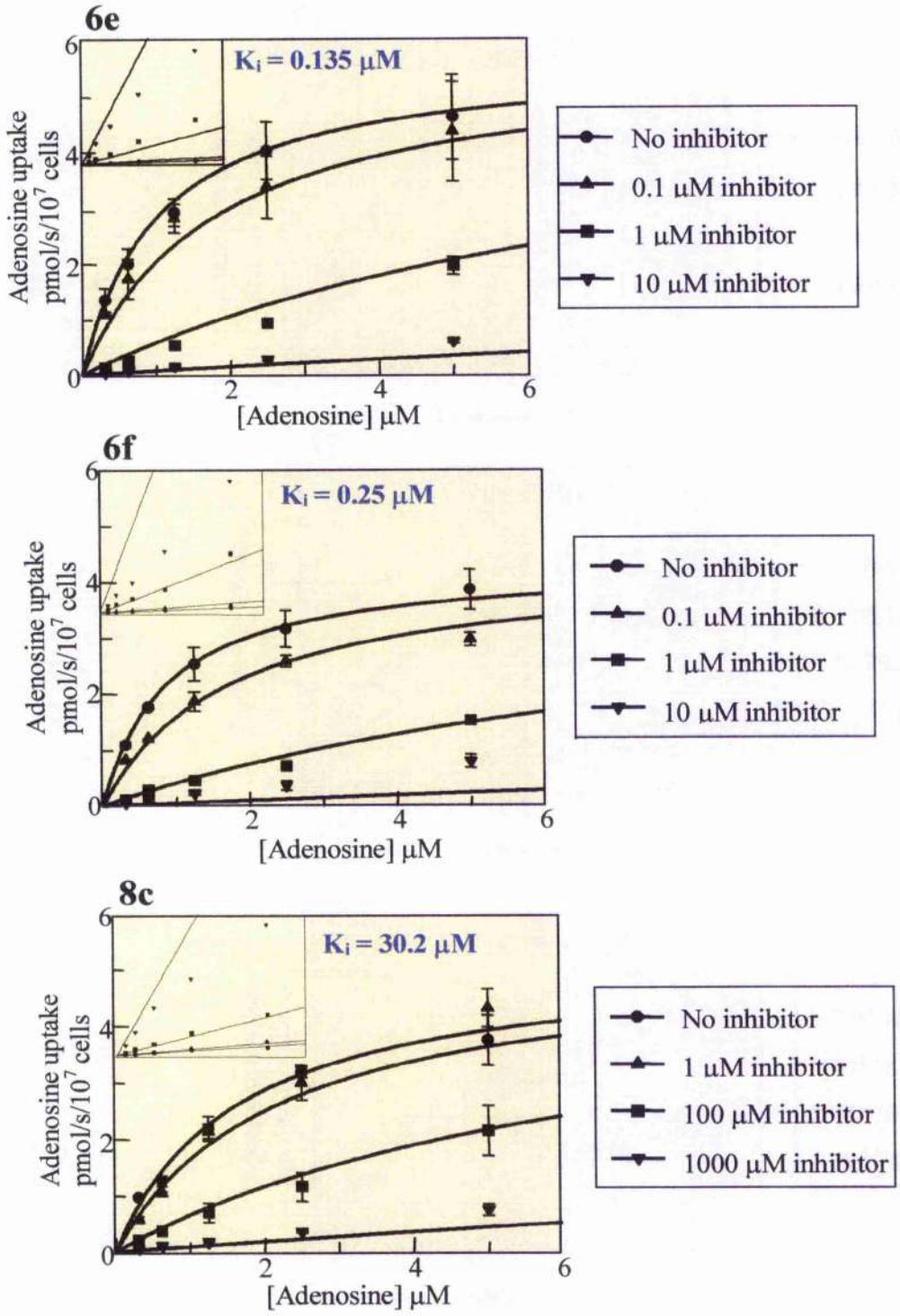


Figure 3.5. Examples of Grafit calculated K_i values.

Apparent K_i values were calculated using the competitive inhibition equation from the Grafit 4.0 software. Double reciprocal plots are shown as inserts.

The double reciprocal Lineweaver Burke plots as illustrated in Figure 3.5 (inserts) are typical in that a downward slope is apparent in the data points for the lowest concentrations of substrate. This result could be due to contamination of the samples. Another possibility is that the presence of multiple adenosine transporters in the trypanosomes has affected the data. However, the experiments were carried out in the presence of 1 mM inosine (which blocks the P1 transporter) and with adenosine concentrations at or below 5 μM. Under these conditions the amount of adenosine that enters the cells through components other than P2 has been shown to be negligible (de Koning & Jarvis 1999), which does not agree with the multiple transporter theory. The reason for the downwardly sloping plots has not been further investigated due to time constraints.

The series of compounds 5a-5c show an increase in apparent affinity for the P2 transporter associated with an increase in chain length. Non-polyamine-like core units such as in 5d-f result in a decrease in affinity. It is not certain why the polyamine analogues, or their central chain length, should be important in the affinity of the compounds for the transporter. One possibility is that chain length affects the length of time each molecule occupies a transporter's binding site, or the time taken for the carrier to transport the molecule across the cell membrane and return to its native state (see discussion). There is no data to confirm that these compounds actually enter into the cells via P2 since the assay simply measures their ability to inhibit adenosine uptake, making it impossible to speculate on their transport.

In series 6a-h, a comparison of the $(CH_2)_9$ compounds to their corresponding $(CH_2)_{12}$ compounds shows that, as before, an increase in core unit chain length results in an increase in P2 apparent affinity. The increase in apparent affinity is not consistent across the series, being between 4 and 40 fold. Apparent affinity for P2 is consistently greater in the compounds bearing one monomethylated or dimethylated melamine amine on each transport unit than for their unmethylated counterparts, 5b and 5c. Any more methyl groups added to the remaining amine group results in a decrease in apparent affinity. This decrease in apparent affinity when both amine groups are methylated is as expected as the addition of methyl groups acts to disrupt the primary P2 recognition motif, $H_2N–C(R_1)=N(R_2)$.

The unexpected increase in apparent affinity in the monosubstituted compounds 6a and 6e as compared with 5b and 5c may be due to the effect of the methyl groups on the π-orbitals within the triazine ring. As suggested in work published by de Koning (de Koning & Jarvis 1999), a high binding affinity for the P2 transporter requires an aromatic system on the substrate. It is proposed that the π-orbitals of the substrate stack with the π-orbitals of an aromatic residue within the substrate binding site to increase the binding energy of the interaction and stabilise the bond. This π-orbital stacking has been shown to play a role in other systems, such as in the binding of purines to the purine nucleoside phosphorylase (PNP) (Koellner et al. 1998) and phosphoribosyltransferase (Vos et al. 1998) of *Escherichia coli*. The forces between two interacting π-systems can reach 10 kJ/mol or more under optimal conditions (Hunter et al. 1991) and so contribute greatly to the affinity of a substrate for an enzyme.

It is possible that the methyl substitution of one amine group might increase the electron density in the compound's π-system. This could potentially increase the contribution of π-stacking to the binding of the compound, while leaving one amine group open as the primary recognition motif for the transporter.

The series 7a–e consists of polyamine analogues attached to just one melamine ring, resulting in an asymmetrical compound. Only one compound in this series contains a 9 carbon chain (7a), the rest have 12 carbon chains. The measured apparent affinity of 7a is greater than that of its comparable 12 carbon compound 7d, their apparent K_i values being 17 μM and 25 μM respectively. This is contrary to the general trend, but the difference is less than two fold and is therefore not a substantial difference in apparent K_i value. The monosubstituted monomethylated compound 7b has the greatest affinity for P2, fitting in with the previously established pattern in series 6. The monosubstituted dimethylated compound 7c, and the disubstituted dimethylated compound 7e have the next highest affinity for P2, their apparent K_i values being 1 μM and 0.6 μM respectively.

The series 8a-f follows the previous trend in that the 12 carbon chain compounds show greater affinity than the 9, and that methylated compounds show greater apparent affinity than

unmethylated compounds, although 8e and 8f have similar K_i values. There does not appear to be any advantage to the extra melamine ring attached via an extra methylene bridge, and affinity is reduced when compared to equivalent compounds bearing just one melamine ring.

3.2.2 Trypanocidal Activity *in vitro*

3.2.2.1 Trypanocidal activity against *T. b. brucei* 427

Results for *in vitro* activity are shown in Table 3.1. Examples of IC_{50} graphs are shown in Figure 3.6.

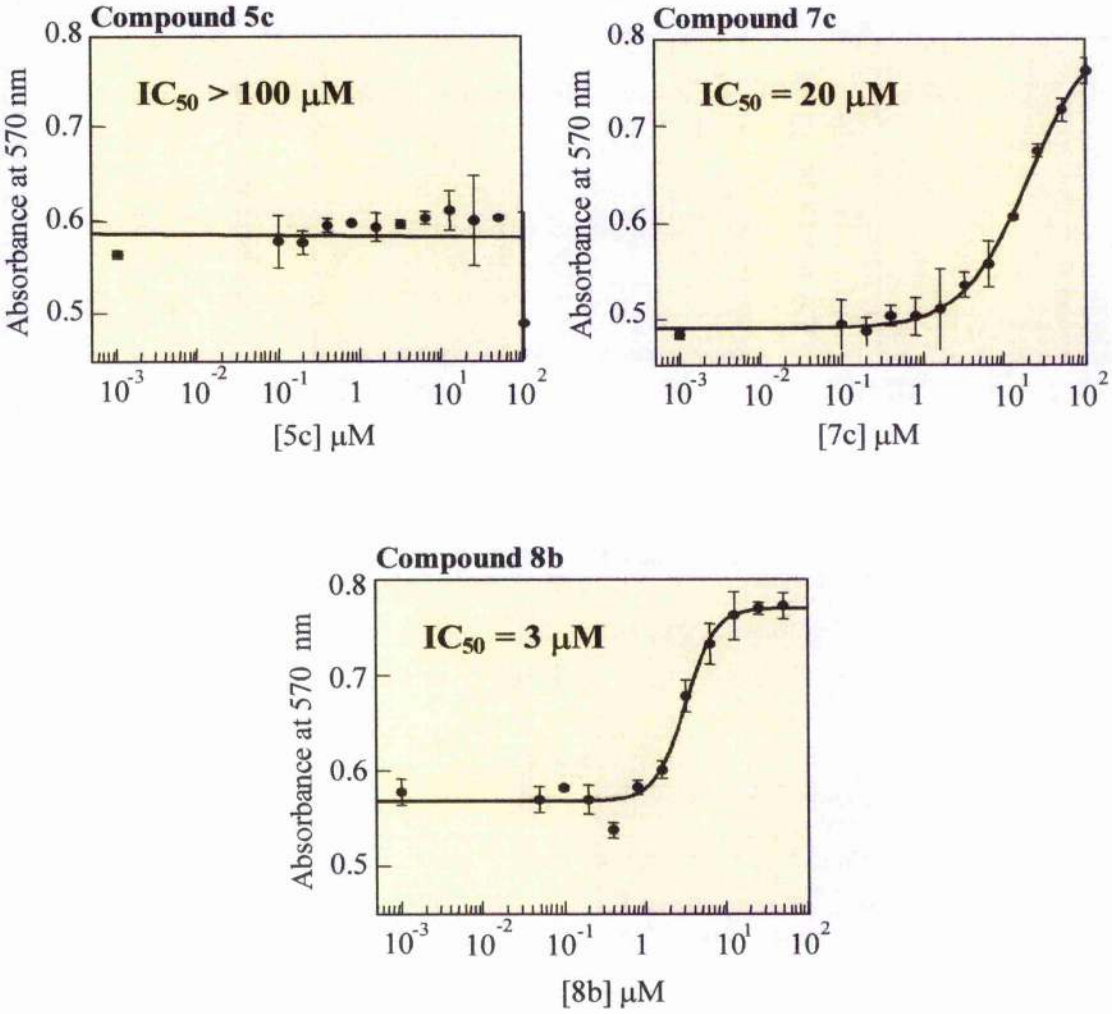


Figure 3.6. Examples of Grafit calculated IC_{50} values.
Absorbance of Alamar Blue at 570 nm was plotted against concentration of drug, and an IC_{50} calculated from Grafit software.

The length, or even the nature, of the central core unit of the compounds tested does not appear to affect the toxicity of that compound. The only general pattern observed is that methylated compounds are more toxic. In general, the greater the number of methyl groups present on the compound, the greater their trypanotoxicity. Most of the compounds tested showed insufficient toxicity to be useful as trypanocides. The exceptions to this are 6 c, e and h, and 8 b, c, e and f. These compounds have IC_{50} values in the range of 0.1-4.0 μM.

3.2.2.2 Activity against *ΔTbat1* (P2 knock out) cells

The experimental compounds 6e, 8b, 8c and 8e were tested for activity against *T. b. brucei* strain MiTat 1.2 (BS221), and a *ΔTbat1* null mutant constructed from this line which lacks P2 activity (Enock Matovu, unpublished data). If the compounds are taken up into the cells via the P2 transporter, it was anticipated that the mutant cell line lacking the transporter would display some degree of resistance as compared to the parent cell line. The results are shown in Table 3.2.

Compound	Toxicity in vitro IC_{50} (μM)		Resistance Factor
	TbAT1 cells	*ΔTbat1* cells	
6e	114	74	0.64
8b	2.9	4.7	1.62
8c	0.9	0.9	1
8e	0.6	0.9	1.5

Table 3.2. *In vitro* activity against *TbAT1* and *ΔTbat1* cells.
Resistance factors represent the differences in activity of the compounds in different cell lines.

All of the compounds show very little or no difference in toxicity between *TbAT1* and *ΔTbat1* cell lines. This suggests that although the compounds do interact with the P2 transporter to inhibit adenosine uptake, the toxicity observed is not dependent on uptake of these compounds via the P2 transporter. The differences in toxicity exhibited between these cell lines and the *T. b. brucei* 427 line are most likely to be due to strain differences.

3.2.3 *In vivo* Activity

Based on their apparently high affinity for P2 and their low IC_{50} *in vitro*, as well as a degree of selective toxicity towards trypanosomal cells as compared to L-6 rat myoblast cells *in vitro* (Table 3.3), compounds 6e, 8b, 8c and 8e were selected for testing *in vivo*.

Compound	*T. brucei* 427 IC_{50} (μM)	*T. b. rhodesiense* IC_{50} (μM)	*T. cruzi* IC_{50} (μM)	L-6 rat myoblast cells IC_{50} (μM)
6e	2.0	9.8	116	nd
8b	3.0	0.265	54.8	177
8c	0.1	0.265	76.8	177
8e	2.0	0.44	>77	105

Table 3.3 Comparative toxicities of 6e, 8b, 8c and 8e.
T. b. rhodesiense STIB 900, T. cruzi Tulahuen C4 and L-6 rat myoblast cell data supplied by Reto Brun, Swiss Tropical Institute, Switzerland.
nd = not defined.

It was found, however, that a dose of 1 mg/kg was not curative, and a dose of 10 mg/kg induced severe adverse reactions in mice, precluding further investigation.

3.3 Discussion

In the above results apparent K_i values have been taken as an indication of the affinity of a compound for the P2 transporter. In experiments, K_i values for these compounds were obtained by measuring the degree to which they inhibit the uptake of a different, labelled, substrate for the same transporter. Relating K_i values measured in this manner to the inhibiting substrates' affinity for the transporter should be done with caution. A K_i measured in this way is dependent on the amount of time that the inhibiting compound is associated with a transporter and is blocking the uptake of labelled substrate. This is in turn influenced by the time taken for the inhibitor to travel across the membrane and be released on the other side, and how long it takes for the transporter to reconfigure itself to be able to receive another molecule. The longer each inhibitor molecule is associated with the transporter and blocking the uptake of the labelled substrate, the lower the K_i will appear. This means for example that a compound with a low affinity that travels across the membrane slowly may appear to have the same K_i as a compound that has a high affinity but a faster turnover. Notwithstanding the

limitations of inhibition studies as a means to infer affinity, such studies are in wide use and can be used as a basis to measure affinity.

The results for these polyamine analogues show that the addition of a P2 recognition motif has produced a series of compounds which block adenosine uptake via the P2 transporter and in some cases show toxicity *in vitro*. There is no indication as to whether any of these compounds are actually transported into the cell via P2, only that they associate with the transporter in some way. Neither is there any correlation between the toxicity of a compound and its interaction with the P2 transporter. Additionally, a *ΔTbat1* cell line, which lacks P2 activity, gained no advantage over a wild type cell line in toxicity studies. This implies that transport via P2 is not critical for the uptake of these compounds into the cell or for their toxic effects. Other mechanisms of uptake, interactions with intracellular targets or toxic effects caused by interactions at the cell surface are probably more important in determining toxicity. This concurs with the findings of Tye et al on a smaller series of related compounds (Tye et al. 1998).

Although a few of the compounds are highly toxic to *T. b. brucei in vitro*, neither their intracellular target(s) nor their mechanisms of action are known. Toxicity could potentially be an artefact of the breakdown of the polyamine analogues outside the cell, and the toxicity of their breakdown products. Polyamine oxidase, present in bovine serum, has previously been shown to mediate trypanolytic activity when incubated with exogenous polyamines (Traore-Leroux et al. 1987). The polyamines spermidine and spermine (Table 3.1) were shown to be toxic *in vitro* at concentrations less than those inside trypanosomes, but in the same concentration range as the polyamine analogues. Polyamine oxidase could also potentially break down the polyamine analogues to toxic by products that affect cell viability *in vitro*.

The quantity of product supplied did not allow for a study of breakdown products. An attempt to investigate the toxicity of the separate transport unit portion of the compounds was made using free melamine rings, but these were found to have very poor solubility in aqueous solution making it impossible to measure their toxicity with any accuracy.

Chapter IV

The Activity of Various P2 Recognition Motif Bearing Compounds as Targeted Trypanocides

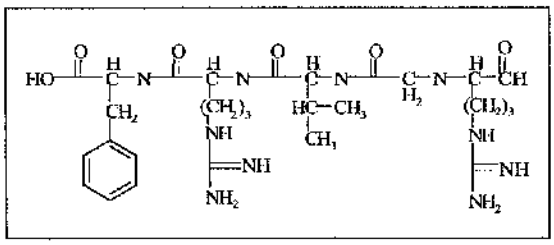
4.1 Introduction

Further to the work detailed in Chapter III, three more groups of potentially trypanocidal compounds, all of which carry the P2 recognition motif, were tested for their abilities to interact with the P2 transporter and their toxicity to *T. b. brucei* 427. The first group of compounds was selected with the assistance of Dr. R. A. Hill (Department of Chemistry, University of Glasgow), from The Combined Chemical Dictionary on CD-ROM version 5.2, Chapman and Hall, CRC Press, 2002. They were identified from the database by searching for the primary P2 recognition motif, H_2N–$C(R_1)$=$N(R_2)$ within the structure of the compounds.

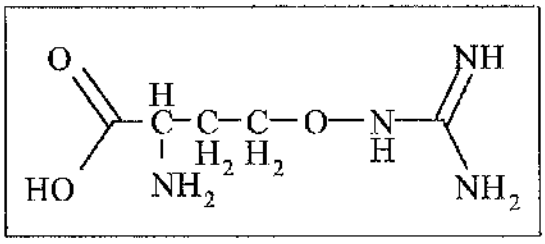
The HIV gp120 binding mimetics, and the nitrofuran and nitroimidazole groups of compounds were synthesised by Burkhard Klenke, Cyrille Boussard and Gorka Jimenez-Bueno at the Welsh School of Pharmacy, Cardiff University.

4.1.1 Database Compounds

Out of the many compounds identified, 6 were chosen on the basis of their likely toxicity and, importantly, their availability. These compounds are described and illustrated below.

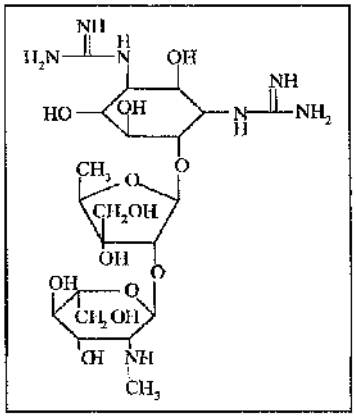


Antipain. An oligopeptide antibiotic originally isolated from *Streptomyces* spp. Antipain is commonly used as a protease inhibitor, inhibiting trypsin-like serine and some cysteine proteases, but has also been shown to have some effects in suppressing experimental carcinogenesis.

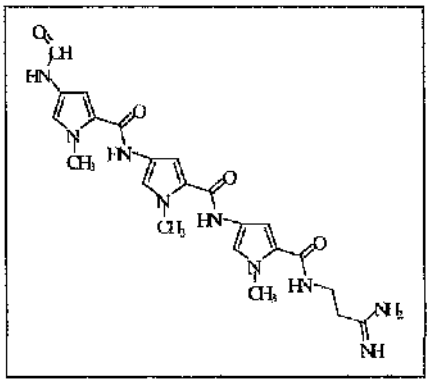


Canavanine. Originally isolated from the Jackbean, this compound is found in the seeds of three subfamilies of leguminous plants. It has insecticidal properties, and has additionally been

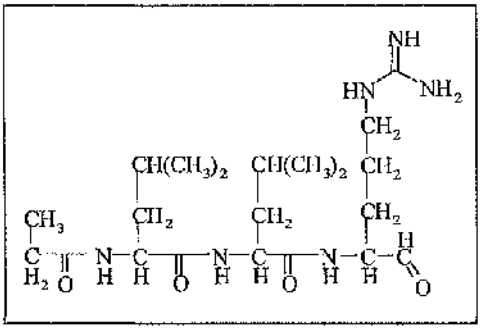
shown to have antineoplastic, antibacterial and antifungal properties.



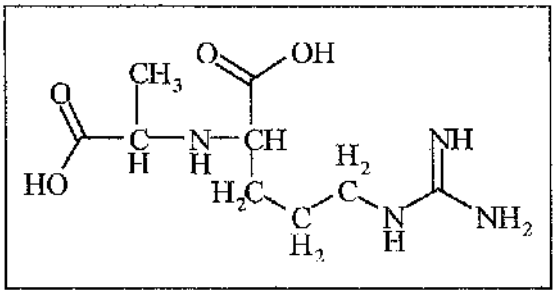
Dihydrostreptomycin. Obtained by the reduction of streptomycin, this aminoglycoside antibiotic was originally isolated from *Streptomyces humidus*. It is a broad spectrum antibiotic, but has been restricted to veterinary use due to likely ototoxicity.



Distamycin A. This antiviral agent is used topically to treat herpes simplex infections. It is a peptide antibiotic isolated from *Streptomyces distallicus*.



Leupeptin. As for Antipain, Leupeptin is commonly used as a protease inhibitor, inhibiting trypsin-like serine and some cysteine proteases. It is a peptide antibiotic produced by many *Streptomyces* spp, and shows antiplasmin and anti-inflammatory activity.



Octopine. Octopine is isolated from aqueous extracts of the tentacle muscles of *Loligo pealii* and *Octopus vulgaris*, as well as from the adductor muscles of the scallop *Pecten magellanicus* and the muscles of the octopod *Eledone moschata*.

4.1.2 HIV gp120 Binding Mimetics

As the name suggests, these compounds were originally designed for a separate project that aimed to stop HIV invasion of a host cell. A peptide library was constructed which mimicked the CD4 marker on the cell surface with which the HIV protein gp120 interacts. Following this interaction, gp120 undergoes a conformational change and interacts with a chemokine receptor. Another HIV protein, gp41, now penetrates the cell membrane and fusion takes place (Boussard et al. 2002). Having discovered some small peptides that mimicked CD4, further simplified compounds were made which incorporated the most important groups on CD4 for the interaction.

These compounds carry the P2 recognition motif in the form of a benzamidine group, with the exception of WSP 670, which has a melamine substitution (Figure 4.1), and 509 and 510 which carry guanidine groups. This afforded us the opportunity of investigating the feasibility of using a benzamidine motif in order to target compounds to the P2 transporter.

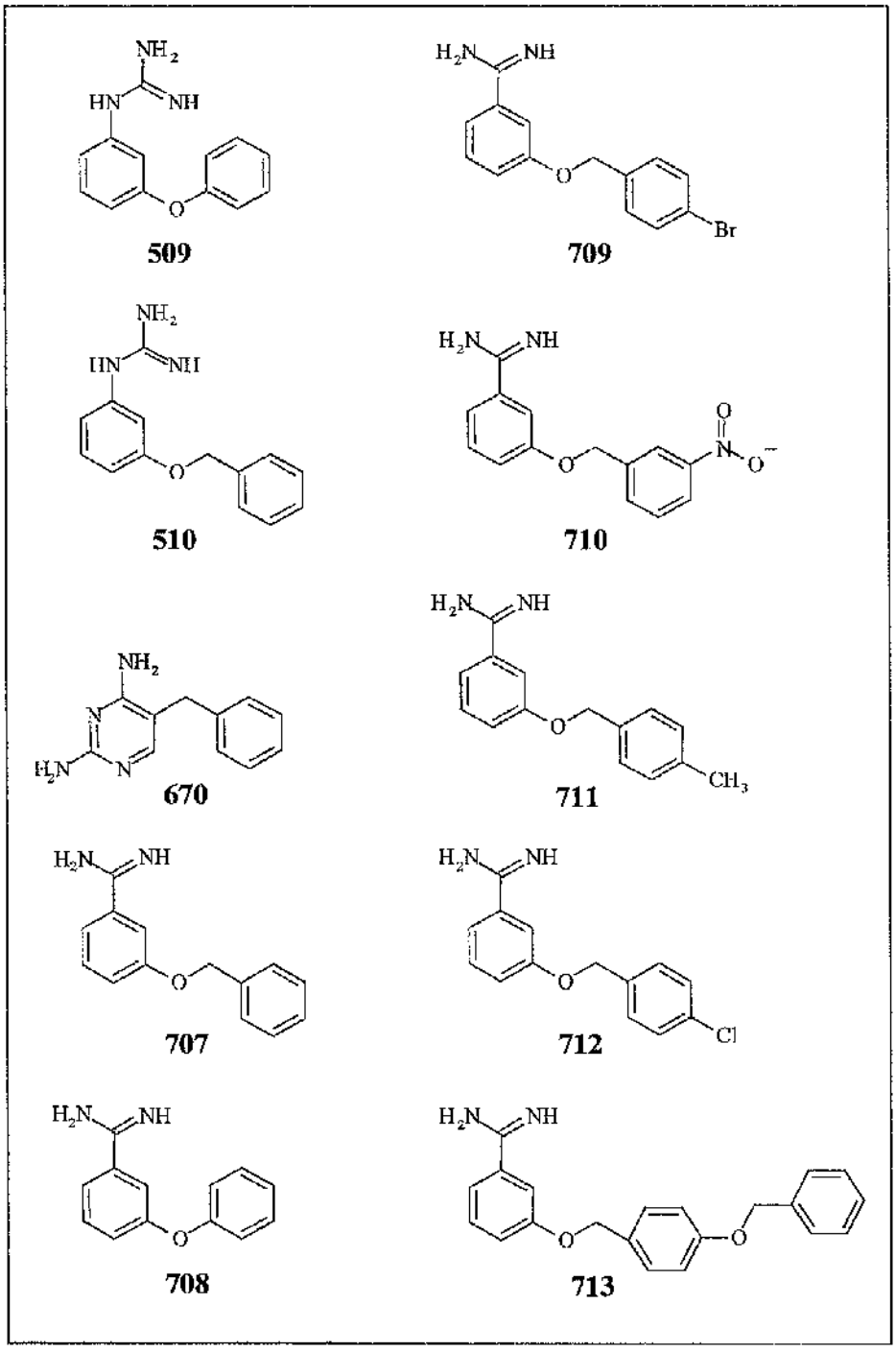


Figure 4.1. Structures of HIV gp120 binding mimetics.

4.1.3 Nitrofuran and Nitroimidazole Compounds

Nitroheterocyclic compounds are broad-spectrum antimicrobial agents, ordinarily used in the treatment of infections caused by anaerobic protozoa and bacteria. Nitrofurans are commonly used in human and veterinary medicine to treat giardiasis (Boreham et al. 1991), typhoid (Carcelen et al. 1989) and cholera (Rabbani et al. 1991). The 5-nitrofuran, Nifurtimox is used to treat infections of *Trypanosoma cruzi*, the causative agent of Chagas disease (DoCampo et al. 1981). Nifurtimox has also been suggested for use in cases of melarsoprol-refractory *T. brucei* infections (Pepin et al. 1992).

The 5-nitroimidazole compound, Metronidazole is used to treat *Giardia intestinalis*, *Trichomanas vaginalis* and *Entamoeba histolytica*, and also as a treatment for, or as a prophylactic against, many anaerobic bacterial infections (Townson et al. 1994). The 2-nitroimidazole, Benznidazole is used to treat Chagas disease (Rodriques & de Castro 2002). More recently Megazol, a 5-nitroimidazole, has been shown to have activity against *T. cruzi* (Filardi & Brener 1982), and is also effective in clearing experimental *T. brucei* infections when used with suramin or melarsoprol (Enanga et al. 1998).

The activity of the nitroheterocyclic compounds is mainly attributed to the reduction of their nitro group to form the nitro anion free radical ($NO_2^{\cdot -}$). This reactive species may then exert its toxicity by interacting with parasite macromolecules, or by reacting with oxygen to produce reduced oxygen by-products such as $O_2^{\cdot -}$ or H_2O_2 that could cause oxidative stress within the cell. Trypanosomatids are more vulnerable to oxidative stress as they lack catalase and glutathione peroxidase, and rely on the parasite-specific enzyme trypanothione reductase to maintain an intracellular reducing environment (Fairlamb & Le Quesne 1997; Krieger et al. 2000).

Nitroheterocyclic compounds exhibit a range of side effects, which are the result of mammalian cells also having the ability to reduce nitroheterocyclic compounds to release toxic metabolites. The side effects are limited, however, by the increased ability of

mammalian cells to protect themselves from oxidative stress and, perhaps, by changes in selectivity of nitroreductase activities in cells.

Selective uptake into the parasite, in principle, offers another means of ensuring activity against the trypanosome but not the host cells. A P2 recognition motif has been added to our test compounds with the intention of targeting the compounds more specifically to trypanosomes and in an attempt to reduce some of the toxic side effects in the host.

The nitrofuran compounds in this group carry the P2 recognition motif in the form of a melamine group (Figure 4.2, compounds 719, 744 and 745), substituted in the same way as the polyamine analogues described in the previous chapter. Compounds 746 and 747 consist of the melamine transport unit alone, with no nitro group (Figure 4.2). Compounds, 748 and 749, which have no recognition motif for P2, were included as controls (Figure 4.2). The nitroimidazoles carry either a benzamidine motif occluded by a *t*-butanoic acid group (Figure 4.2, compounds 817 and 836), or an open benzamidine (Figure 4.2, compounds 854 and 855).

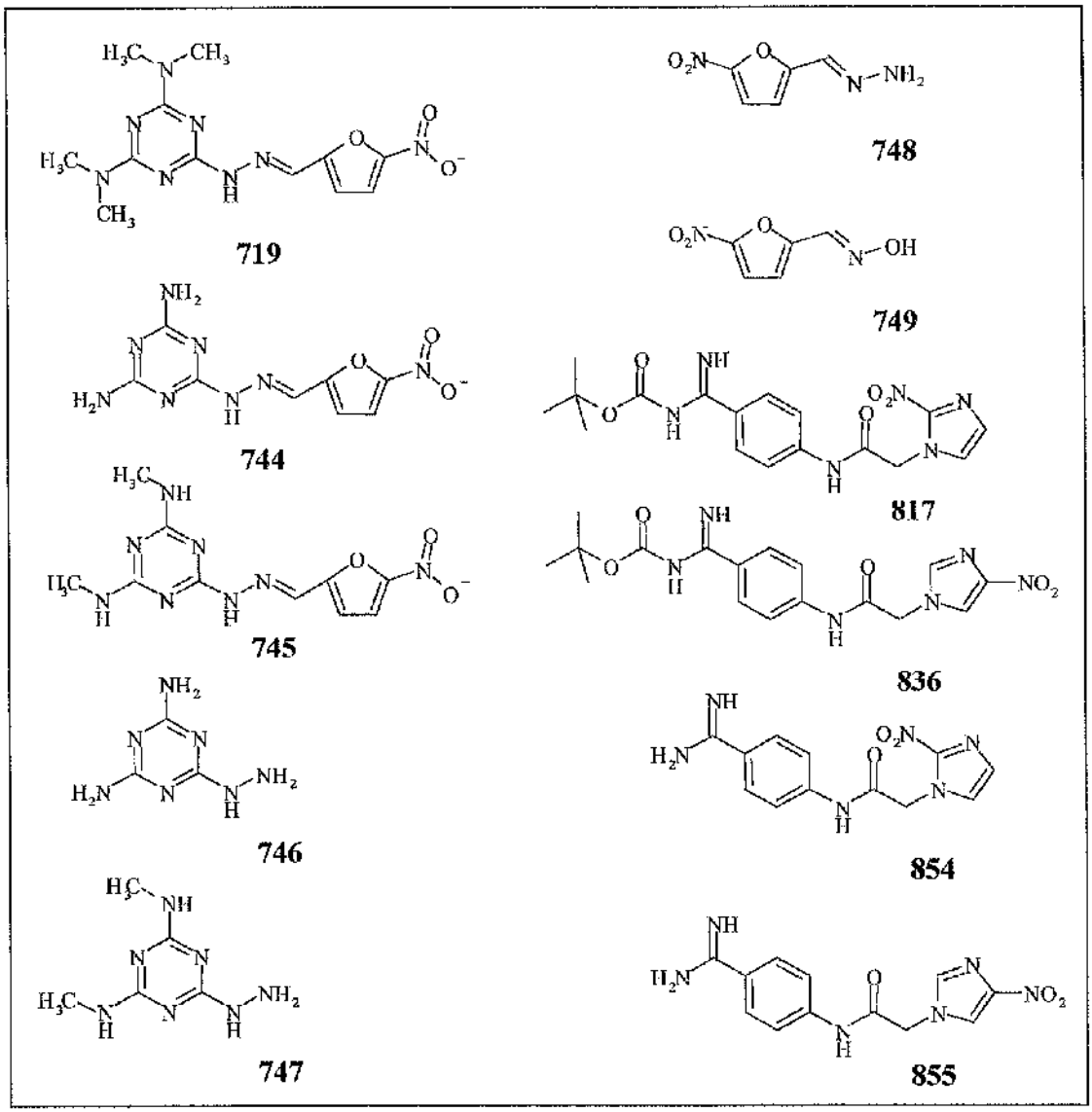


Figure 4.2. Structures of Nitrofuran and Nitroimidazole Compounds.

4.2 Results

An IC_{50} for the inhibition of adenosine uptake was measured for all compounds in order to determine the optimum range of adenosine concentrations to use in subsequent experiments to derive apparent K_i values. If compounds showed little promise of P2 interaction at this stage, it was not deemed necessary to continue the uptake experiments.

4.2.1 Database Compounds

The results for these compounds are summarized in Table 4.1.

Compound	Inhibition of adenosine transport, IC_{50}.	Toxicity *in vitro*
Antipain	No inhibition at 1 mM	None at 200 μM
Canavanine	No inhibition at 1 mM	None at 200 μM
Dihydrostreptomycin	No inhibition at 1 mM	None at 200 μM
Distamycin A	10.6 μM	None at 200 μM
Leupeptin	No inhibition at 1 mM	None at 200 μM
Octopine	No inhibition at 1 mM	None at 200 μM

Table 4. 1. Inhibition of adenosine transport and toxicity of database compounds.
All the above experiments were carried out on the *T. brucei* 427 cell line.

The only compound to show any interaction with P2 was Distamycin A. With the exception of Dihydrostreptomycin and Distamycin A, all the compounds lack any aromatic ring structure which plays an important part in forming attachments with the transporter (de Koning & Jarvis 1999). The large size and bulky structures of these compounds may further contribute to their lack of interaction with the P2 transporter.

Also of note is that of all compounds, Distamycin A, is the only compound to carry the P2 recognition motif in the form of an amidine group. The motif on the other compounds occurs as part of a guanidine group. For reasons detailed in the following section, this structural group reduces affinity for the P2 transporter. Additionally, Distamycin A carries its amidine group at the end of a carbon chain, which allows it more flexibility in movement, and may

assist it in associating with the transporter. That Distamycin A should be able to inhibit adenosine uptake via P2 to a moderate extent is apparent, but a lack of toxicity *in vitro* made further investigation redundant.

4.2.2 HIV gp120 binding mimetics

Results for these compounds are shown in Table 4.2.

Compound	Inhibition of adenosine transport, IC_{50} (μM)	Toxicity *in vitro*. IC_{50} (μM)			Resistance Factor
		427	*TbAT1*	*ΔTbat1*	
509	9.3	57	52	79	1.52
510	8.05	109	111	128	1.15
670	0.38*	96	97	258	2.65
707	0.38	54	33	44	1.33
708	0.38	56	37	60	1.62
709	0.81	10	6	7	1.06
710	0.33	66	58	71	1.22
711	0.21	14	10	28	2.80
712	1.01	13	11	14	1.27
713	1.57	7	8	12	1.50

Table 4.2. Inhibition of adenosine transport and toxicity of HIV gp120 binding mimetics.
*The figure for 670 is a calculated apparent K_i value.

4.2.2.1 Interaction with the P2 Transporter

All of the above compounds, with the exception of 509 and 510, which carry a guanidine group, have very high apparent affinities for the P2 transporter. Only a handful of the polyamine analogues showed apparent affinities in the same range and the nitrofuran and nitroimidazole group has no compounds within 10-fold of this range of affinities.

Compounds 509 and 510 are identical to compounds 708 and 707 respectively, except they carry a guanidine group in place of the amidine. This factor alone apparently accounts for 24-fold and 21-fold differences in apparent affinity for P2. This may be explained by the spatial positioning of the P2 recognition motif with respect to the benzene ring. The primary nitrogen of the guanidine in compounds 509 and 510 is pyramidal in structure whereas the amidine groups on the benzamidine compounds are planar. The P2 recognition motif in a guanidine

group is held rigidly and at such an angle as to make association with the P2 transporter molecule more difficult. The spatial differences are illustrated in Figure 4.3.

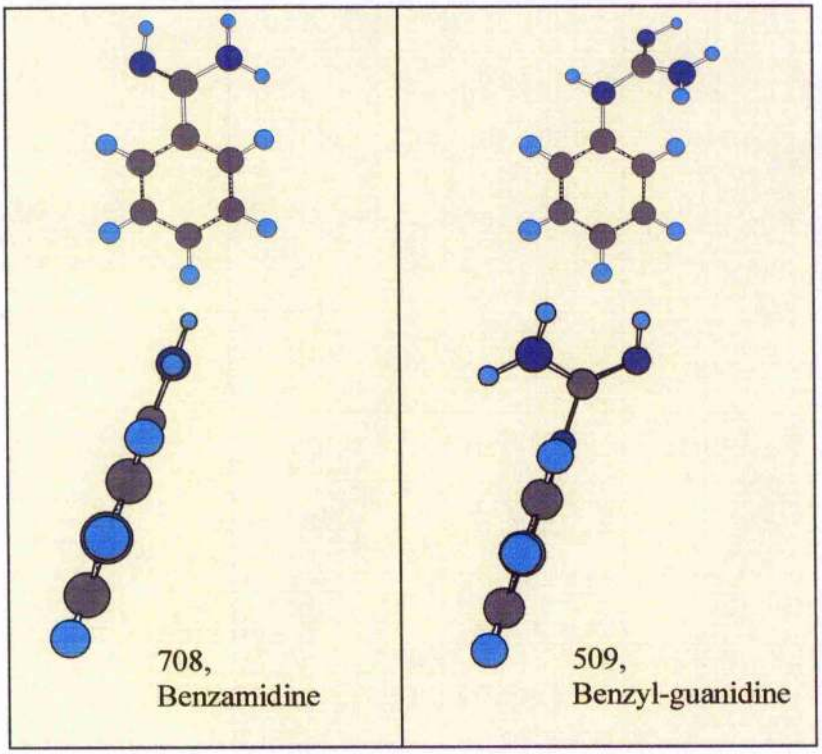


Figure 4.3. Spatial configuration of the P2 recognition motifs of 708 and 509.
The guanidine is pyramidal and holds the amidine portion at a set angle to the benzene ring. The amidine group in the benzamidine is planar.

Compounds 670, 707 – 713 all exhibit IC_{50} values in the range of 0.38 – 1.5 μM which, on average, are higher affinities for P2 than have been measured for compounds from the other groups in this study. The benzamidine and benzene groups in compound 708, in association with the electronegative oxygen atom, might be able to form a conjugated π-system, which in turn might contribute to affinity. However, the energy attributable to a conjugated π-system cannot account for such a large increase in apparent affinity. Furthermore, compound 707, which has an additional carbon atom between the 2 benzene rings, has the same affinity for P2 as does 708 even though a conjugated π system is unlikely. The same can be seen in the diaminopyrimidine 670 which, lacking the electronegative oxygen between the benzene rings to contribute to a conjugated π system, is also unlikely to have this extended system.

The compounds 709 and 712 have an addition halogen attached to the end benzene ring, and this appears to very slightly reduce their apparent affinities for P2. Why the additional halogen should affect their interactions with P2 is unknown. Compound 713 also has a lower apparent affinity for P2. With three benzene rings in this compound, it may be the bulk of this compound that is interfering with substrate/transporter interactions.

4.2.2.2 Trypanocidal Activity *in vitro*

Results are listed in Table 4.2.

All of the compounds displayed disappointingly low toxicities to *T. brucei* 427 *in vitro*. This precluded any *in vivo* trials. However, because the very high apparent affinities for P2 displayed by the compounds might represent a high specificity for uptake via P2 they could be interesting lead compounds. In order to investigate this further the compounds were tested against the P2 transport deficient *ΔTbat1* cell line, and its parent cell line, *TbAT1*, that has a normal P2 adenosine transport phenotype.

At best the compounds showed a 2.6-fold resistance factor, most being well under 1.5-fold less active in the *ΔTbat1* cell line. This result would indicate that the compounds do not rely solely on the P2 transporter to gain access into the trypanosome cell. There may be other transporters involved, for example the high and low affinity pentamidine transporters (HAPT1 and LAPT1), or the compounds may enter the cells by diffusion.

That diffusion may be involved is given more strength when considering the lipophilicity of the compounds. They all contain two very hydrophobic benzene residues, and those with the least difference in toxicity between *TbAT1* and *ΔTbat1* cell lines, 709 and 712, are those that contain the halogen extensions which, carrying very little charge, might also contribute to lipophilicity. If this were the case, any high specificity for P2-mediated uptake that the compounds may have, would be concealed by the diffusion component in this three-day experiment.

709 and 712 are the most toxic compounds in this group, along with 713 carrying three benzene rings. Apart from a potential increase in lipophilicity in these compounds, and therefore an increased rate of entry into the cell, the reasons for their increased toxicity are not clear.

4.2.3 Nitrofuran and Nitroimidazole Compounds

Results for these compounds are shown in Table 4.3.

Compound	K_i or IC_{50} (μM)	Toxicity *in vitro*. IC_{50} (μM)			Resistance Factor
		427	*TbAT1*	*ΔTbat1*	
719	129	0.4	0.2	0.3	1.5
744	53.4	0.36	0.41	0.66	1.60
745	4.6	6.7	11.9	14.8	1.24
746	11.9*	None at 200 μM			
747	59.3	None at 200 μM			
748	404	10.7	23.5	13.7	0.58
749	No inhibition at 1 mM	2.6	1.1	1.2	1.09
817	13.09	62.7	111	119	1.07
836	3.65	None at 200 μM			
854	1.58	97.8	> 200	191	
855	2.88	None at 200 μM			

Table 4.3. Inhibition of adenosine transport and toxicity of nitrofuran and nitroimidazole compounds.
*This value is an IC_{50} value.

4.2.3.1 Interaction with the P2 Transporter

Within the nitrofuran group, 745, with one methyl group attached to each melamine amine, has the highest apparent affinity for P2. 744, which carries an undisturbed P2 recognition motif, has an apparent affinity 11 times less than 745. This pattern of increased affinity in compounds bearing monomethylated amine groups is also seen in the polyamine analogues described in Chapter III. The addition of more methyl groups in 719 acts to further disrupt the P2 recognition motif and the apparent affinity of this compound for P2 drops considerably.

746 and 747 do not carry any nitro groups, but are included in this group as they are the transport recognition portions of the compounds 744 and 745. These compounds behave in the

opposite way to the nitrofurans and the polyamines in that 747, with the monomethylated melamine amines, has less affinity for P2 than 746 with an open primary P2 recognition motif. The 2-nitrogen chain may be involved in some interactions with P2, but no further explanation of this unexpected behaviour presents itself. 748 and 749 lack any P2 recognition motif at all, and as such they have very little or no affinity for P2.

The nitroimidazoles carry benzamidine groups as their P2 recognition motifs. 817 and 836 have a *t*-butanoic acid motif attached to the benzamidine amine group, and 854 and 855 respectively are their *t*-butanoic acid free derivatives.

The compounds, 817 and 836, with the disrupted P2 recognition motifs are less able to associate with the transporter than their respective benzamidines, 854 and 855. They do, however, have moderate affinities for P2, signifying that they are still able to associate with the transporter, even with the *t*-butanoic acid group attached to the benzamidine ring.

4.2.3.2 Trypanocidal Activity *in vitro*

In terms of toxicity the nitrofuran compounds were very much more active (100-fold or more) than the nitroimidazoles. Toxicity is not dependent on the presence of a functional P2 transporter as compounds were as toxic to P2 deficient *ΔTbat1* cells as to *TbAT1*. Also, 748 and 749, without any P2 recognition motif, were just as toxic as 744 and 745, again indicating that uptake via the P2 transporter is not critical in the toxic actions of these compounds. There is a possibility that the compounds may bypass any active transport system and gain access into the cell by diffusion. Other nitroheterocyclic compounds have already been shown to act in this way, e.g. Megazol (Barrett et al. 2000) and Nifurtimox (Tsuhako et al. 1991).

The compounds 744 and 745 were carried forward into *in vivo* trials.

4.2.3.3 Trypanocidal Activity *in vivo*

744 and 745 were tested in ICR mice at concentrations of 40 mg/kg. The compounds were introduced intraperitoneally on the second and third days after infection with *T. b. brucei* 427. The dose was restricted by the amount of compound available for testing.

Treatment with 744 resulted in four animals in the study group clearing infection temporarily from the bloodstream. These animals relapsed 7-14 days later and were sacrificed. One animal stayed clear of infection for up to 40 days (Table 4.4). A dose of 40 mg/kg was well tolerated, with no apparent side effects. It remains possible that a higher dose would have been able to clear the infection in all the study animals.

745 showed no *in vivo* activity (Table 4.4) at the dose used.

Group protocol		Parasitaemia																				
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		40
744 40 mg/kg ip.	1	0	0	< 6.9	< 6.9	0	0	0		0		< 6.9	7.5									
	2	0	0	< 6.9	< 6.9	0	0	0		0		< 6.9	0				< 6.9		8.1			
	3	0	0	< 6.9	< 6.9	0	0	0		0		0	0	< 6.9			< 6.9		8.1			
	4	0	0	< 6.9	< 6.9	0	0	0		0		0	< 6.9				0		7.8	8.7		
	5	0	0	0	< 6.9	0	0	0		0		0					0		0	0		0

Group protocol		Parasitaemia																				
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		40
745 40 mg/kg ip.	1	0	0	< 6.9	7.5	8.7																
	2	0	0	< 6.9	7.5	9																
	3	0	0	< 6.9	7.8	9																
	4	0	0	0	7.5	8.7																
	5	0	0	< 6.9	7.5	9																

Group protocol		Parasitaemia																				
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		40
Diminazene aceturate 20 mg/kg ip.	1	0	0	0	0	0	0	0		0		0				0				0		0
	2	0	0	0	< 6.9	0	0	0		0		0				0				0		0
	3	0	0	< 6.9	0	0	0	0		0		0				0				0		0
	4	0	0	< 6.9	< 6.9	0	0	0		0		0				0				0		0
	5	0	0	0	0	0	0	0		0		0				0				0		0

Group protocol		Parasitaemia																				
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		40
No treatment	1	0	0	< 6.9	7.8	8.4																
	2	0	0	< 6.9	7.5	8.7																
	3	0	0	< 6.9	7.8	8.7																
	4	0	0	0	7.5	8.4																
	5	0	0	< 6.9	7.5	8.4																

Table 4.4. Results of *in vivo* drugs trails with nitrofuran compounds 744 and 745.

Numbers 1-5 in each group represents an individual animal within that group. Parasitaema was measured daily using the rapid matching method (Herbert & Lumsden 1976), the numbers are equivalent to the log number of cells / ml of blood. Cells representing live animals are shaded yellow, dead animals are shaded red.

4.3 Discussion

That a guanidine group is less effective for targeting compounds to the P2 transporter is apparent from the above results. Guanidine compounds were among the first drugs to be designed to treat trypanosomiasis, Synthalin (decamethylene diguanidine, Figure 4.4) being the most effective. At therapeutic levels they were thought to reduce glucose levels in the bloodstream of the host, and so starve the parasitic cells of this essential nutrient (Schoenbach & Greenspan 1948). It was quickly discovered, however, that diamidine compounds, such as N-undecane 1-11 diamidine (Figure 4.4), were more effective and less toxic (King et al. 1938). In 1942 the amidine groups were further substituted for the aromatic benzamidine group (Ashley et al. 1942) to eventually produce pentamidine, which is still used in trypanosome chemotherapy today. This group also observed that any slight change in the benzamidine ring reduced the toxicity of their compounds.

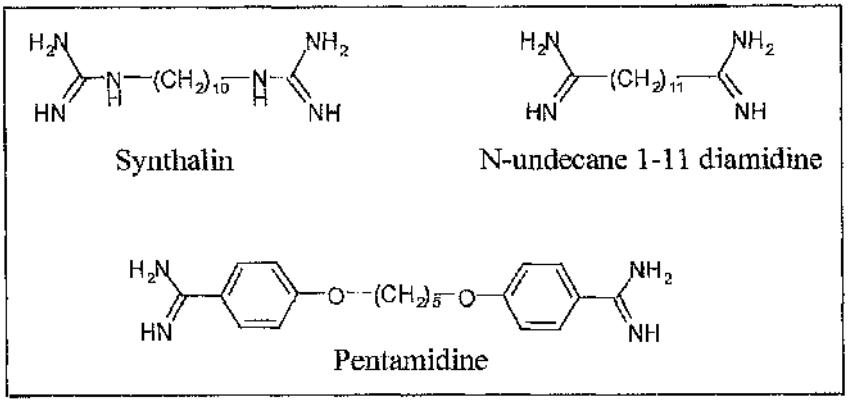


Figure 4.4. The structures and evolution of diamidine trypanocides.
Guanido compounds were superseded by the diamidines, which in turn led to the development of aromatic diamidines.

The loss in affinity for P2 may be attributable to the rigidity of the P2 recognition motif in the guanidine compounds, and its position relative to the benzene ring. The amidine portion is held rigidly at an angle to the benzene (Figure 4.3). The P2 recognition motif and the π orbitals are now on different planes, which could either shield the recognition motif from the transporter, or physically block π stacking interactions, or indeed both.

The benzamidine compounds in this study also show a 10-fold greater affinity for P2 than most of the melamine bearing compounds. 670 is an exception to this, as are 6 of the polyamine compounds, these being 5c, 6a, 6e, 6f, 7b and 7e, which are discussed in the preceding chapter. Interesting to note is that pentamidine, which carries a benzamidine motif, has a higher apparent affinity for P2 (K_m = 0.26 μM) than does one of P2's natural substrates, adenosine (K_m = 0.92 μM) (de Koning 2001a). This could indicate a structural preference for the benzamidine group in P2 interactions. If this were the case then future compounds would benefit from carrying a benzamidine in place of a melamine.

Some support for this idea comes from the nitrofuran and nitroimidazole compounds. The benzamidine compounds 854 and 855 show higher apparent affinities than the melamine compounds 744, 745 and 719. This has to be interpreted with caution, however, as the active groups on these compounds are not comparable, the benzamidines carrying imidiazole groups and the melamines carrying furan groups.

Moreover, the *in vitro* toxicity experiments involving the *TbAT1* and P2 transport deficient *ΔTbat1* cells reveal no difference in toxicity for these compounds towards either cell line, suggesting that the P2 transporter is not critical in the delivery of these compounds. It is possible that these compounds can be carried into the cell via diffusion. Both the HIV gp120 binding mimetics and nitroheterocylic compounds carry very lipophilic residues, which could aid in their passing across the cell membrane. If this is the case, then active transport is indeed unnecessary for uptake. Furthermore, other nitroheterocyles such as Megazol (Barrett et al. 2000) and Nifurtimox (Tsuhako et al. 1991) have been shown to enter trypanosomatid cells by diffusion.

More evidence for the redundancy of the P2 transporter comes from 748 and 749, which lack the P2 recognition motif altogether and consist solely of the nitrofuran portion of 744 and 745. They had extremely little, or no affinity, for P2 whatsoever, but they exhibit toxicities comparable to 744 and 745 *in vitro*. The very slight interaction of 748 with P2 (K_i = 404 μM) might be due to an interaction with the non-melamine amine group on this compound. The

activity of 746 and 747 may also have been affected in some way by the presence of this amine.

The melamine bearing compounds described here have greater affinities for P2 when the melamine amines were monomethylated, as do the polyamine analogues in the previous chapter. This may be related to the methyl substitution increasing the electron density in the compound's π-system, which could potentially increase the contribution of π-stacking to the binding of the compound to P2. In the case of 746 and 747, which represent the carrier portion only, the reverse seems to apply, i.e. the presence of methyl groups diminishes apparent affinity. Other than the possible involvement of the non-melamine amine, no reason for this unexpected behaviour presents itself.

The *in vitro* toxicity experiments clearly show that the nitrofurans are much more toxic to trypanosomes than the nitroimidazoles. As mentioned earlier, the activity of these compounds relies on their nitro group being reduced to an active nitro anion radical. This is in turn related to the reduction potential of their nitro groups. The lower their redox potential, the less likely they are to be reduced. In general, nitrofurans are more easily reduced than nitroimidazoles (Tocher 1997). Anaerobic cells have a lower redox potential than aerobic cells, which contributes to the generally greater toxicity of nitroimidazoles for anaerobic cells. As nitrofurans are more easily reduced than nitroimidazoles, their different redox potentials could also explain the differences in their respective toxicities. African trypanosomes are generally considered to be obligate aerobes, and therefore less able to reduce nitro imidazoles. The efficacy of nitroimidazoles such as Megazol is likely to be due to the presence of a specific enzyme – possessing Megazol-reductase activity – rather than a highly reductive intracellular environment.

The compounds 744 and 745 were tested *in vivo*, the only structural difference between 744 and 745 being the monomethylated melamine amines present on 745. This difference is sufficient to lead to 745 being inactive *in vivo* up to the highest concentration used (40 mg/kg), and 744 being able to clear infection for up to 40 days in one of the animals in the study. The *in vitro* evidence seems to indicate that the melamine groups are not involved in the toxic nature of these compounds, but *in vivo* they must have some effect, whether that is in uptake

or in interactions with intracellular targets. Reto Brun at the Swiss Tropical Institute, Brunel, is taking this promising result further with *in vivo* trials against both acute and chronic trypanosome infections in mice.

Difficulties may arise in any future development of these nitroheterocyclic compounds for clinical use due to mutagenic and carcinogenic properties. Many nitroheterocycles give a positive Ames test, including Megazol (Ferreira & Ferreira 1986), the development of which has been halted for this reason (Poli et al. 2002). Nitrofuran compounds are, if anything, likely to be even more mutagenic as they are more easily reduced to form active metabolites. Nevertheless, 744 shows promise as a trypanocide, and could be a useful lead compound. Further work is needed on this compound to assess its mode of action and its usefulness as a trypanocidal drug *in vivo*.

Chapter V

The Molecular Basis of Drug Resistance in *Trypanosoma equiperdum*

5.1 Introduction

Although the P2 transporter may have some use as a carrier of trypanotoxic compounds, the loss of this transporter has been linked with drug resistance in both laboratory derived drug resistant strains and field isolates (Brun et al. 2001). In order to further investigate the role of P2 in drug resistance, the molecular basis for the development of drug resistance in a diamidine resistant strain of *T. equiperdum* with a much reduced P2-like adenosine transport component has been investigated. Because of the residual adenine sensitive, adenosine transport (P2-like) activity in this strain, it seemed likely that the change in P2 activity was due to a loss in affinity for the drugs as substrates, rather than a loss in P2 function (Barrett et al.1995).

5.1.1 *Trypanosoma equiperdum*

The trypanosomatid, *Trypanosoma equiperdum* infects equines causing a condition known as Dourine (Stephen 1986). The most notable feature of *T. equiperdum* is its inability to be transmitted via an insect vector, instead the parasite is transmitted during coitus. This tissue parasite then localises to the capillaries of the mucous membranes in the urogenital tract and is transferred via these membranes or in seminal fluid during sexual intercourse. Trypanosomes do occasionally appear in the bloodstream of an infected host in chronic infections, which could result in mechanical transfer by a bloodsucking insect moving rapidly between hosts, but this is considered a rare event (Stephen 1986).

Infection with *T. equiperdum* results in a range of clinical symptoms. Early infection is characterised by oedema of the genital organs and a swelling of the lower abdomen. More established infections are generally linked with fever, anaemia, weight loss and the development of neurological symptoms. A typical feature of infection is the development of plaques in the skin. Although many of these symptoms may occur in any infected animal they are not progressive in their order of appearance, and some may not occur at all (Stephen 1986).

Infections with *T. equiperdum* share many symptoms with the closely related blood parasite *Trypanosoma evansi*. Positive diagnosis therefore relies upon the identification of parasites in the oedematous fluids of an infected animal. Trypanosomes can be few and far between, even in these tissues, so diagnosis often relies upon indirect methods such as immunosorbent assays (Alemu et al. 1997) or DNA probes (Zhang & Baltz 1994). Treatment can be delivered in the form of suramin, berenil or cymelarsan (Brun et al. 1998), however clinical trials documenting efficacy have not been conducted, and the treatment of stallions and brood mares with available compounds is not recommended (Stephen 1986).

The incidence of Dourine is difficult to determine due to the lack of veterinary resources in endemic areas. However due to its lack of reliance upon an insect vector and climatic conditions it has a very wide distribution. Historically the disease is thought to have originated in Africa and to have spread with animal movements to become endemic in most of Europe. From here the disease spread east and was carried across to the Americas. Dourine was a disease of great concern over all of North America at the turn of the 20th century. It was eradicated from Canada in the 1920's but persisted in the U.S.A. until 1949. Dourine is currently found in Africa, Asia, Eastern Europe and South and Central America (Stephen 1986).

Phylogenetically *T. equiperdum* was thought to have originated from the mechanically transmitted parasite *T. evansi*. Although both species are related to *T. brucei*, they are more closely related to each other. *T. equiperdum* was thought to have descended from *T. evansi* because of its more specialised method of transmission and smaller host range (equines only as opposed to equines, camels, buffalo, cattle and deer in *T. evansi* infections) (Brun et al. 1998). Molecular data in the form of isoenzyme analysis (Lun et al. 1992a) and RFLP analysis of kinetoplast DNA as well as genes for ribosomal RNA and variant surface glycoproteins (Lun et al. 1992b), supports the idea that the two are the same species.

Recently, however it has been suggested that in fact *T. evansi* is derived from *T. equiperdum*. This theory is based on the presence of maxicircles in the kinetoplast DNA of *T. equiperdum* (Frasch et al. 1980; Riou & Saucier 1979) which are absent in *T. evansi* (Borst et al. 1987). As it seems unlikely that *T. equiperdum* could have re-acquired maxicircles, it has been proposed

that *T. evansi* arose from a *T. equiperdum* cell that had lost its maxicircles and acquired the ability to reside in the general bloodstream allowing mechanical transmission (Lun et al. 1992b).

5.1.2 *Trypanosoma equiperdum* BoTat 1

5.1.2.1 Selection for Drug Resistance

T. equiperdum BoTat 1 (Bordeaux trypanozoon antigenic type 1), here referred to as *T. equiperdum* P originated from a stock held at the Pasteur Institute, Paris. A berenil resistant line, *T. equiperdum* PBR was derived from *T. equiperdum* P by serial passage through mice treated with increasing sub-curative doses of berenil (Zhang et al. 1993).

T. equiperdum PBR is 35.6-fold less sensitive to berenil and 4-fold less sensitive to cymelarsan than the sensitive line *in vitro*. The resistant line is also insensitive to berenil up to the maximum tolerated dose in Swiss mice and 4-fold less sensitive to cymelarsan *in vivo* (Table 5.1).

Cell Line	*in vitro* (MEC_{100})		*in vivo* (CD_{100})	
	Berenil (μg/ml)	Cymelarsan (μg/ml)	Berenil (mg/kg)	Cymelarsan (mg/kg)
T. equiperdum P	0.0556	0.005	18	0.5
T. equiperdum PBR	1.78	0.02	>89	2

Table 5.1. Drug sensitivities of *T. equiperdum* P and PBR (Zhang et al. 1993).
MEC_{100} = Minimal effective concentration which killed 100% of trypanosomes during 24 hours of drug exposure.
CD_{100} = Curative dosage in 100% of mice.

5.1.2.2 P1 and P2 Adenosine Transport in *T. equiperdum* P and PBR

The P2 purine nucleoside transporter has been shown to be important for the transport of melaminophenyl arsenicals and diamidines in trypanosomes (Carter & Fairlamb 1993). Its loss of function through loss or mutation of *TbAT1* confers resistance to these drugs (for a full

discussion see Chapter I). Barrett et al. investigated the role that this transporter plays in the resistance of *T. equiperdum* PBR to trypanocidal drugs (Barrett et al. 1995).

The sensitive strain, *T. equiperdum* P, had a typical P1/P2 adenosine transport system. The P1 component was found to have an apparent K_m of 0.6 μM and a V_{max} of 8.4 pmol/s/10^8 cells for adenosine. The P2 component had an apparent K_m of 0.7 μM and a V_{max} of 6.9 pmol/s/10^8 cells. This accounts for all measurable adenosine uptake into these cells.

In *T. equiperdum* PBR the overall rate of adenosine uptake was reduced. The P1 transport component has a K_m of 0.66 μM and a V_{max} of 4.15 pmol/s/10^8 cells, making it comparable to that in *T. equiperdum* P although with a slightly reduced V_{max}. After blocking P1 with excess inosine, a P2-like adenosine transport component, which could be inhibited by the addition of excess adenine, was apparent. However, apparent K_m and V_{max} values could not be determined for this component.

Because of this residual P2-like activity, it was postulated that the change in P2 activity was due to a loss in affinity for the drugs as substrates. Drug resistance was acquired very quickly (T., Baltz, personal communication) suggesting that any change might have come about by the appearance of one or more point mutations in the *TbAT1* gene. This mechanism of drug resistance, where point mutations to a transporter have altered the substrate specificity and conferred drug resistance, has been reported in *Leishmania donovani* (Vasuvedan et al. 2001).

5.2 Results

5.2.1 Rapid Fluorescence Test for Drug Resistance

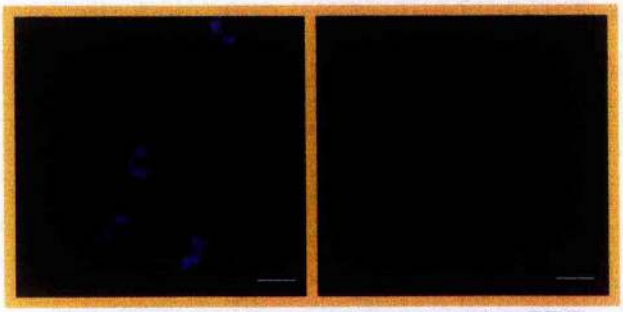
As described in Chapter VI, the diamidine compounds DB 75 (2,5-bis (4-amidinophenyl) furan) and DB 544 (2,5-bis (4-amidinophenyl) 2,3-bis methylfuran) enter trypanosomes via the P2 transporter and fluoresce when bound to DNA. The absence of this transporter on a cell surface results in a delay in the development of fluorescence within a cell. *T. equiperdum* P and PBR were tested for their ability to internalise these fluorescent compounds. The time

taken for fluorescence to develop is shown below, in Table 5.2 and a comparison of fluorescence at 10 minutes in Figure 5.1.

Incubation time (min)	DB 75 (10μM)		DB 544 (10 μM)	
	P	PBR	P	PBR
0	+	-	+	-
1	+	-	+	-
2	+	-	+	-
5	+	-	+	-
10	+	-	+	-

Table 5.2. Development of fluorescence, at room temperature, in the presence of 10 μM DB 75/DB 544.

- : no fluorescence
+ : fluorescence



T. equiperdum P *T. equiperdum* PBR

Figure 5.1. Development of fluorescence from DB 75 in *T. equiperdum* P and PBR cell lines after 10 minutes.

Fluorescence is clearly visible in the P cell line, but has not yet developed in the PBR cell line. Magnification is 100x, bars are 10 μm.

The lack of fluorescence developing in the PBR cell line indicates the lack of a typical P2 transport component capable of associating and internalising these diamidines.

5.2.2 Sequencing of *T equiperdum* P and *T. equiperdum* PBR *TbAT1*

Figure 5.1 indicates that the P2 transporter is not functional in *T. equiperdum* PBR, which contradicts the original hypothesis of a P2 transporter carrying point mutations affecting

substrate affinity. It was therefore decided to compare *TbAT1* from both the P and PBR cell lines.

The entire *TbAT1* ORF (1389 bp) and short flanking regions (PCR product size ~1500 bp) were amplified, and 10 independent PCR products sequenced from each cell line in order to identify and confirm the presence of any point mutations in the sequence. DNA for sequencing was extracted from cells grown in mice that had either been challenged with berenil *in vivo* at 20 mg/kg and survived exposure (*T. equiperdum* PBR), or had been shown to be from infections that could be successfully treated with berenil at 20 mg/kg (*T. equiperdum* P).

5.2.2.1 PCR of *TbAT1*

PCR was conducted using the primers P2F and P2R, both of which fall outwith the open reading frame (ORF) of *TbAT1*, Figure 5.2. All PCR reactions were conducted in the presence of *Pfu* polymerase in order to minimise the risk of introducing point mutations into the sequence. PCR product, once purified was cloned in the PGEM-T vector and sequenced. Both strands were fully sequenced in all cases, using internal primers to give complete coverage of the gene.

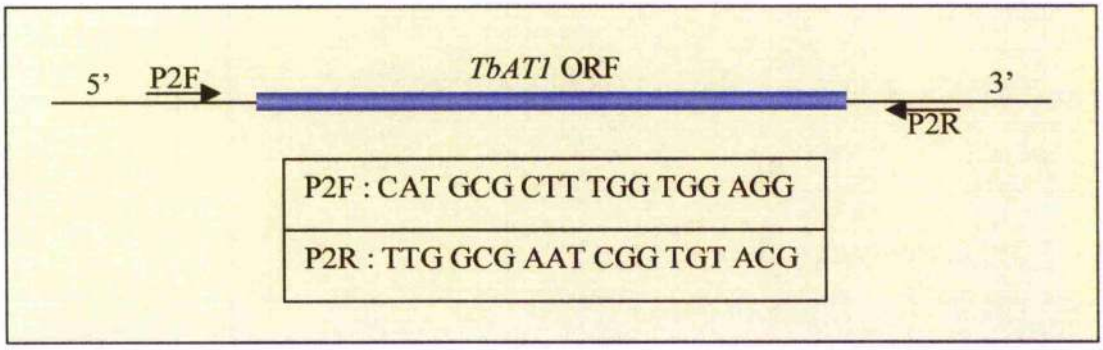


Figure 5.2. *TbAT1* open reading frame and primers used for amplification.

PCR product from this reaction was approximately 1500 bp in length.

5.2.2.2 Sequences

Sequencing indicated that all of the *T. equiperdum* PBR clones have identical sequence, varying from that of *T. brucei* 927 by 6 nucleotide point mutations resulting in 2 changes at the amino acid level. The *T. equiperdum* P sequences were slightly more ambiguous, resulting in 3 different patterns of point mutations, and 3 patterns of amino acid changes, Table 5.3.

On closer examination of the *T. equiperdum* P sequence data, there appeared to be two main patterns. One carries adenosine residues at positions 151 and 627 ('a' allele, clones P6, P8 and P9), which is identical to *T. brucei* 927 strain and the drug resistant PBR cell line. The other pattern, as seen in clones P1-5, P7 and P10, has cytosine residues at these positions ('c' allele). The cytosine point mutation at position 151 results in an amino acid change from a lysine to a glutamine.

Table 5.3. Summary of sequencing in *T. equiperdum* P/PBR.

Sequence identical to that of *T. brucei* 927 strain, is highlighted in light yellow, differences are highlighted in dark yellow.

Strain/ Clone	Position												
	Changes in nucleotide sequence									Changes in amino acid sequence			
	21	151	205	542	625	627	699	716	1122	51	69	181	239
927	C	A	G	G	T	A	T	A	A	K	A	G	D
P1	T	C	G	A	C	C	C	G	G	Q	A	E	G
P2	T	C	G	A	C	C	C	G	G	Q	A	E	G
P3	T	C	G	A	C	C	C	G	G	Q	A	E	G
P4	T	C	G	A	C	C	C	G	G	Q	A	E	G
P5	T	C	G	A	C	C	C	G	G	Q	A	E	G
P6	T	A	G	A	C	A	C	G	G	K	A	E	G
P7	T	C	G	A	C	C	C	G	G	Q	A	E	G
P8	T	A	G	A	C	A	C	G	G	K	A	E	G
P9	T	A	G	A	C	A	C	G	G	K	A	E	G
P10	T	C	A	A	C	C	C	G	G	Q	T	E	G
PBR1	T	A	G	A	C	A	C	G	G	K	A	E	G
PBR2	T	A	G	A	C	A	C	G	G	K	A	E	G
PBR3	T	A	G	A	C	A	C	G	G	K	A	E	G
PBR4	T	A	G	A	C	A	C	G	G	K	A	E	G
PBR5	T	A	G	A	C	A	C	G	G	K	A	E	G
PBR6	T	A	G	A	C	A	C	G	G	K	A	E	G
PBR7	T	A	G	A	C	A	C	G	G	K	A	E	G
PBR8	T	A	G	A	C	A	C	G	G	K	A	E	G
PBR9	T	A	G	A	C	A	C	G	G	K	A	E	G
PBR10	T	A	G	A	C	A	C	G	G	K	A	E	G
		K/Q	A/T	G/E				D/G					

Clone P10, although conforming to the second pattern ('c' allele, a cytosine at positions 151 and 627) has an additional point mutation, not identified in any other sequence, of an adenosine at position 205 instead of a guanosine (Table 5.3). P10 was not re-sequenced, due to time constraints, but, although both strands were sequenced, the likelihood remains that the extra mutation at position 205 in the ORF is an error caused by misincorporation during PCR amplification. A revised table of nucleotide point mutations, that ignores the point mutations common to all *T. equiperdum* clones, P and PBR is shown below, Table 5.4.

	Clone	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	PBR1	PBR2	PBR3	PBR4	PBR5	PBR6	PBR7	PBR8	PBR9	PBR10
Position	151	C	C	C	C	C	A	C	A	A	C	A	A	A	A	A	A	A	A	A	A
	205	G	G	G	G	G	G	G	G	G	A	G	G	G	G	G	G	G	G	G	G
	627	C	C	C	C	C	A	C	A	A	C	A	A	A	A	A	A	A	A	A	A

Table 5.4. Revised table of nucleotide point mutations.
This table ignores any point mutations common to all *T. equiperdum* clones. The mutation at 205 in P10 is likely to be a sequencing error (see text).

From the sequencing data it appears that *T. equiperdum* P is a heterozygote for *TbAT1* carrying both the 'a' and 'c' alleles, while PBR has lost heterozygosity and is homozygous at this locus containing only the 'a' allele. This has been investigated further by Southern blot.

5.2.3 Southern Blot

The restriction enzymes *BfaI* and *BssSI* cut specifically at position 627 within the *TbAT1* gene. *BfaI* cuts where there is an adenosine residue at this position ('a' allele) and *BssSI* where there is a cytosine residue ('c' allele), Figure 5.3. A Southern restriction digest at this site should be diagnostic for the two putative alleles identified through sequencing.

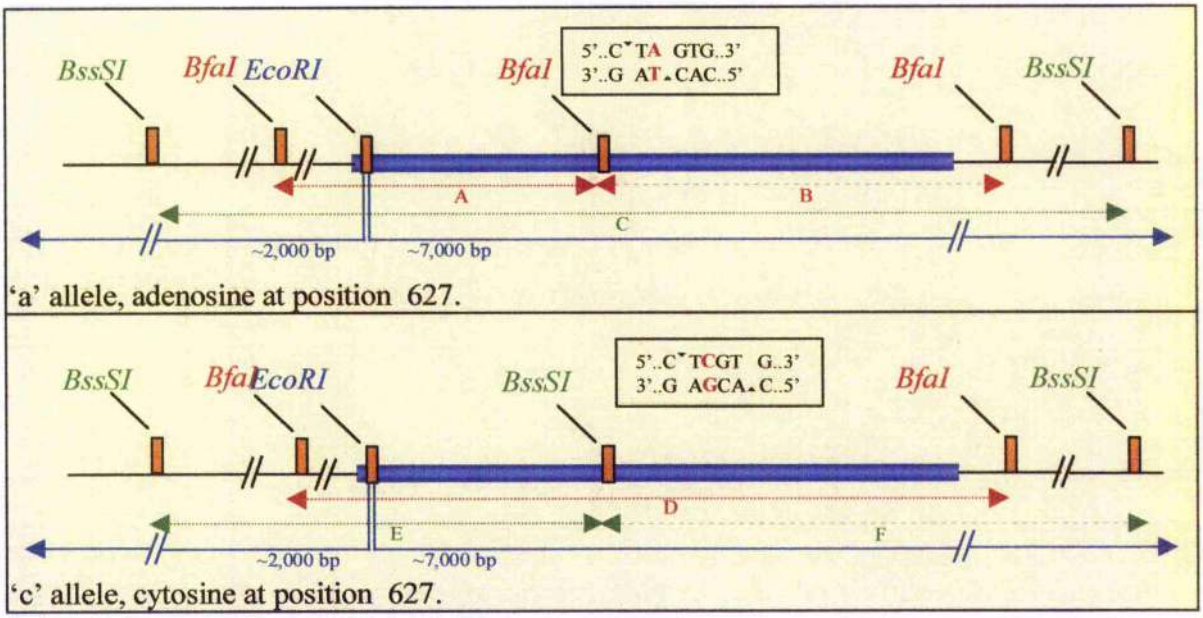


Figure 5.3. Restriction enzyme sites in *TbAT1*.
The blue bar represents the ORF and restriction sites are in orange. The restriction sequences for *BfaI* and *BssSI* are shown, with the sequence differences between the two highlighted in red. The approximate size of the fragments are, A = 10 kb, B = 878 bp, C = 10 kb, D = 4.1 kb, E and F either 9.5 kb or 1.7 kb.

The results of a digestion of genomic DNA with these enzymes and *EcoRI* are shown in Figure 5.4.

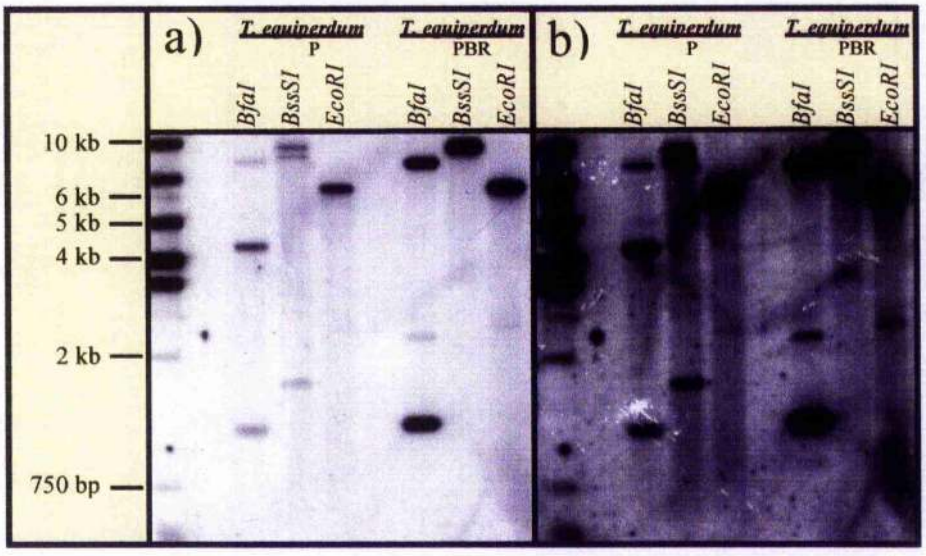


Figure 5.4. Southern Blot of *T. equiperdum* P and PBR DNA.
Genomic DNA was digested with *BfaI*, *BssSI* and *EcoRI*. The Blot was probed with the *TbAT1* ORF. Blot a) is a 2 day exposure, b) is a 2 week exposure of the same blot, showing the presence of the 2 kb band in the *EcoRI* digest of *T. equiperdum* P.

As predicted, the *BfaI* digests, which cut within the 'a' allele only, produce a band of around 900 bp and another, much larger at ~10,000 bp in both P and PBR digests. One other band, of a different size for either sample was identified for each digest. The *T. equiperdum* P digest produced a band of around 4,100 bp. This is likely to be due to an unidentified *BfaI* restriction site upstream of the 'c' allele, closer to the open reading frame than the upstream restriction site in the 'a' allele. The PBR cell line lacks this allele and hence the digest product. The PBR cell line *BfaI* digest has a faint band at approximately 2,000 bp. The significance of this extra band is uncertain. However, it could be kinetoplast DNA, which can frequently be seen in Southern restriction digests carried out with a large quantity of genomic DNA, and runs out at approximately 2,000 bp.

The *BssSI* digests, which cut within the 'c' allele, produce three bands in the P cell line, indicating the presence of two alleles. The PBR cell line digests has only a single band, indicating that this enzyme did not cut the PBR *TbAT1* ORF at all. This points towards the absence of the 'c' allele as predicted from the sequences obtained for PBR.

The *EcoRI* digest produced a band of around 6,000-7,000 bp in both strains. Both cell lines also have a very weak band at around 2,000 bp. This is likely to be an upstream restriction digest product, which contains a 27 bp fragment of the ORF with which the blots were probed.

5.2.4 Northern Blot

RNA isolated from *T. equiperum* P and PBR cell lines, blotted and probed with *TbAT1* (entire ORF) indicates that PBR lacks *TbAT1* transcript (Figure 5.5), while a 2.3 kb transcript corresponding to this gene is apparent in *T. equiperdum* P.

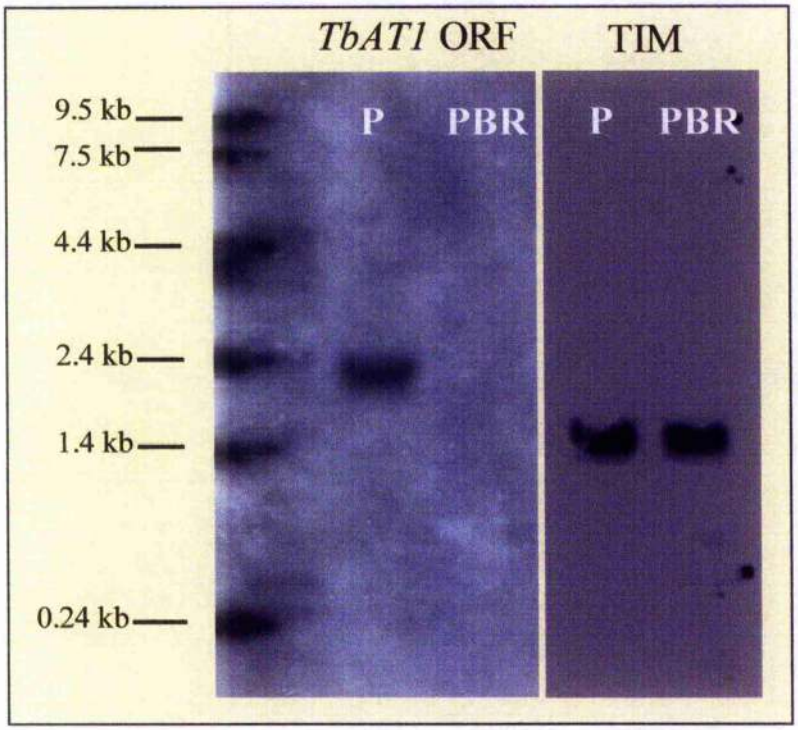


Figure 5.5. Northern Blot of *T. equiperdum* P and PBR.
Blots were probed with the *TbAT1* ORF and subsequently with a control probe for Triosephosphate isomerase (TIM), in order to confirm the presence of RNA in both the P and PBR lanes.

5.2.5 P2 Substrate Uptake Assays

Barrett et al found that in *T. equiperdum* PBR the overall rate of adenosine uptake is reduced. The P1 transport component was slightly less while a P2-like adenosine transport component, which could be inhibited by the addition of excess adenine, was apparent. However, this later component was of low activity and K_m and V_{max} values could not be determined (Barrett et al. 1995).

Because of this residual P2-like activity, it was postulated that the change in P2 activity was due to a loss of affinity for the drugs as substrate molecules. The results presented above, however, indicate a lack of any functional P2 transporter on the surface of *T. equiperdum* due to loss of transcription of this gene. In order to investigate this further, uptake assays were carried out with radiolabelled diminazene aceturate (berenil), previously shown to enter the cell solely through the P2 transporter (H. de Koning, unpublished data). Uptake of 20 μM ^{3}H-berenil over time in both *T. equiperdum* cell lines is shown in Figure 5.6.

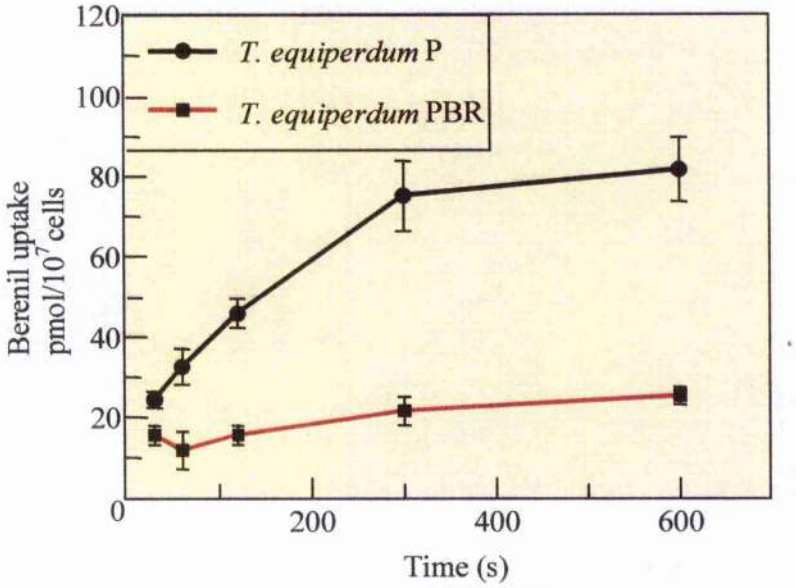
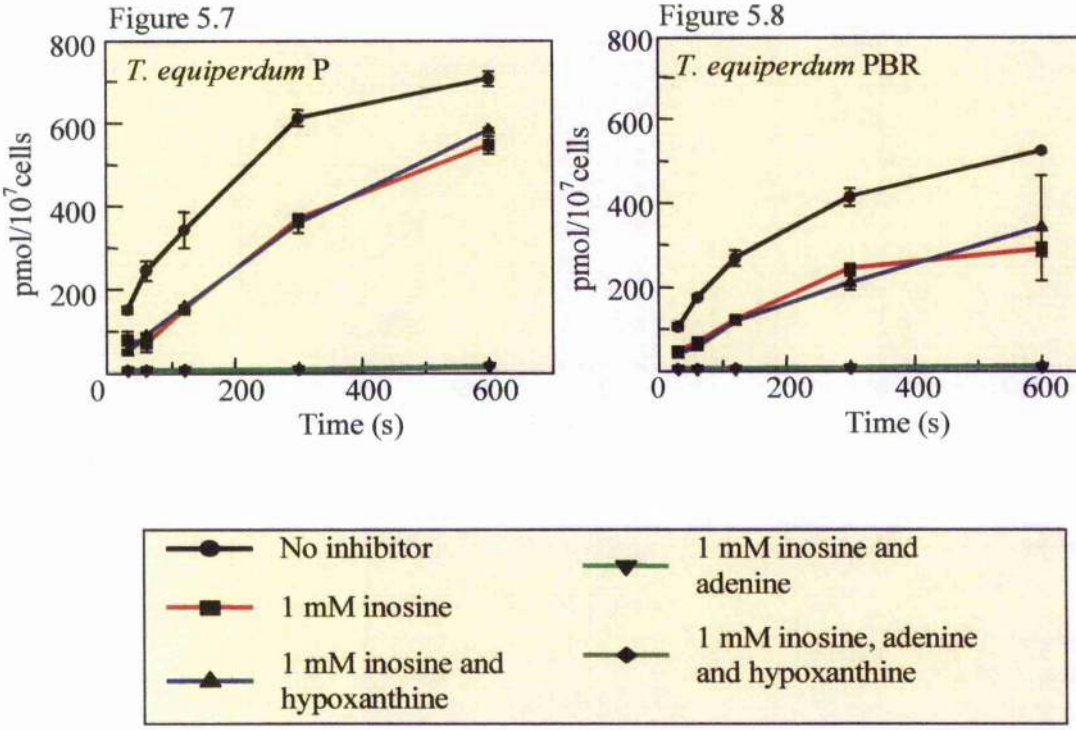


Figure 5.6. Uptake of berenil in *T. equiperdum* P and PBR.
Uptake of 20 μM berenil over time. Bars indicate standard deviations, n = 3.

The results indicate that berenil uptake in *T. equiperdum* PBR is very much reduced. The residual uptake may indicate the presence of another low affinity transporter, or it may be due to ^{3}H-berenil caught in the intercellular space during the experiment.

Since a residual adenine-inhibitable adenosine uptake component had been identified in PBR previously, yet Northern blot data indicated a functional loss of *TbAT1*/P2, the question remained as to the identity of this residual adenine-sensitive adenosine uptake component. Recent research has identified another trypanosome transporter that can convey adenosine with a low affinity. The TbNBT1 transporter has a high affinity for hypoxanthine (K_m= 0.66 μM), and a low affinity for adenosine (K_i = 1900 μM,), as measured when expressed in yeast

(H. de Koning, unpublished data). It was possible that the residual adenosine uptake in *T. equiperdum* PBR was due to the action of this transporter. In order to investigate this the uptake of 10 μM ^{3}H adenosine was followed in the presence of various inhibitors. 1 mM inosine was used to block P1 specific transport, 1 mM adenine to block P2 transport and 1 mM hypoxanthine to block TbNBT1 transport of adenosine. The results are shown in Figures 5.7 and 5.8.



Figures 5.7 and 5.8. Inhibition of adenosine uptake in *T. equiperdum* P and PBR.
Uptake of 10 μM adenosine over time. Bars indicate standard deviations, n = 3.

The above experiments were also carried out on a *ΔTbat1* cell line that lacks any P2 transport function (see Chapter VI for details). The results are shown in Figures 5.9, 5.10 and 5.11.

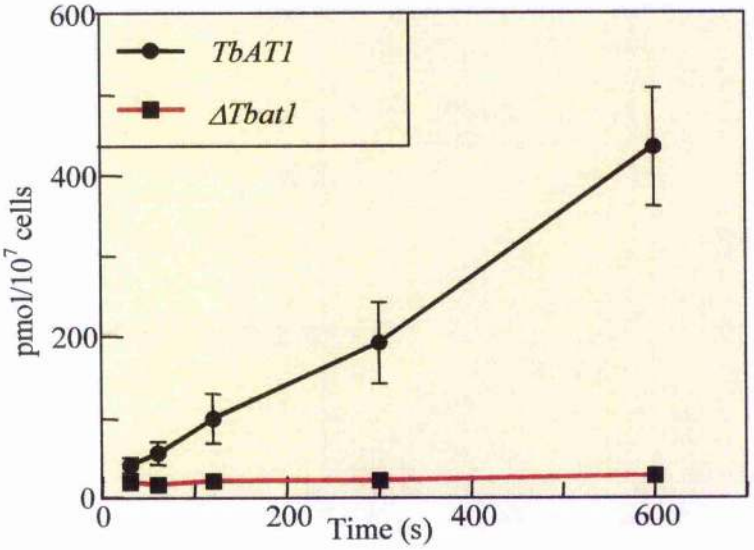


Figure 5.9. Uptake of berenil in *TbAT1* and *ΔTbat1*.
Uptake of 20 μM berenil over time. Bars indicate standard deviations, n = 3.

Figure 5.10

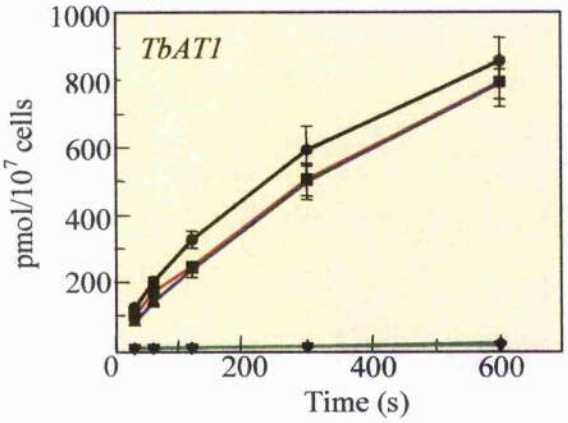
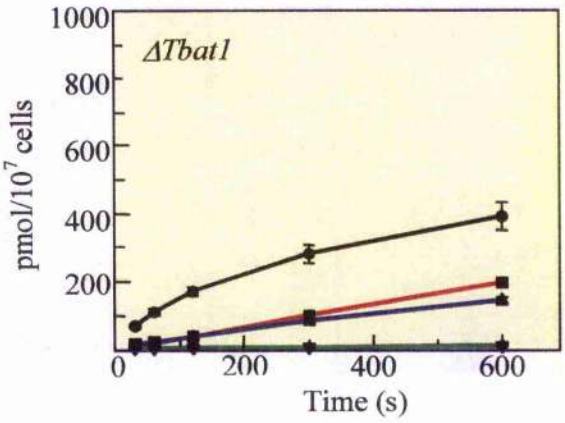
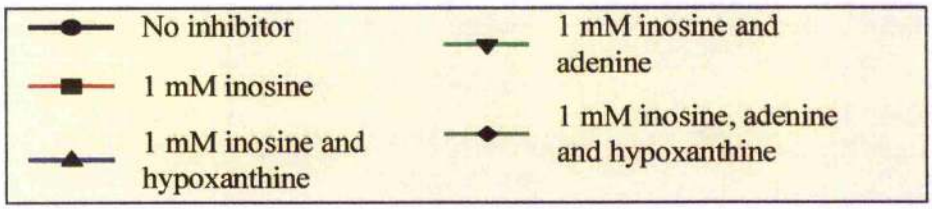


Figure 5.11





Figures 5.10 and 5.11. Inhibition of adenosine uptake in *TbAT1* and *ΔTbat1*.
Uptake of 10 μM adenosine over time. Bars indicate standard deviations, n = 3.

Adenine inhibitable uptake of adenosine was identified in the *ΔTbat1* cell line as well as the PBR cell line. This result indicates the presence of an additional system capable of adenine/adenosine uptake in *T. brucei* and *T. equiperdum*, independent of *TbAT1*. Since previous studies revealed a K_m for adenosine could not be measured for this transporter, it indicates a low affinity for this nucleoside. The transporter is not TbNBT1, as hypoxanthine does not inhibit this adenosine uptake. However, it is probably encoded by one or more of the plethora of other nucleoside/nucleobase transporter genes in the *T. brucei* genome.

5.3 Discussion

TbAT1 in the *T. equiperdum* P cell line differs from that of *T. brucei* 927 by six nucleotide point mutations resulting in two changes at the amino acid level. Two further point mutations, one of which results in an amino acid change, are seen in seven of the clones ('c' allele), the other three having sequence identical, at that point, to *T. brucei* 927 ('a' allele). The *T. equiperdum* PBR cell lines were all identical, having the same sequence as the three *T. equiperdum* P 'a' allele clones.

Of the six mutations seen in all clones, three of these mutations have also been characterised in STIB 777R, a laboratory derived melarsen oxide-resistant cell line (Mäser et al. 1999). Two of these mutations code for changes in the amino acid sequence of TbAT1, and one is a silent mutation. These same three mutations also occur, among others, in *T. b. gambiense* CSF isolates from patients in Uganda, in a *T. b. rhodesiense* isolate (STIB 871) that shows some resistance to berenil and melarsoprol, and in a *T. b. gambiense* stock (K 003) from Angola (Matovu et al. 2001b). Figure 5.12 shows a cartoon of relative position of the mutations in the *TbAT1* ORF in *T. brucei* 927, including those that appear to be conserved between stocks.

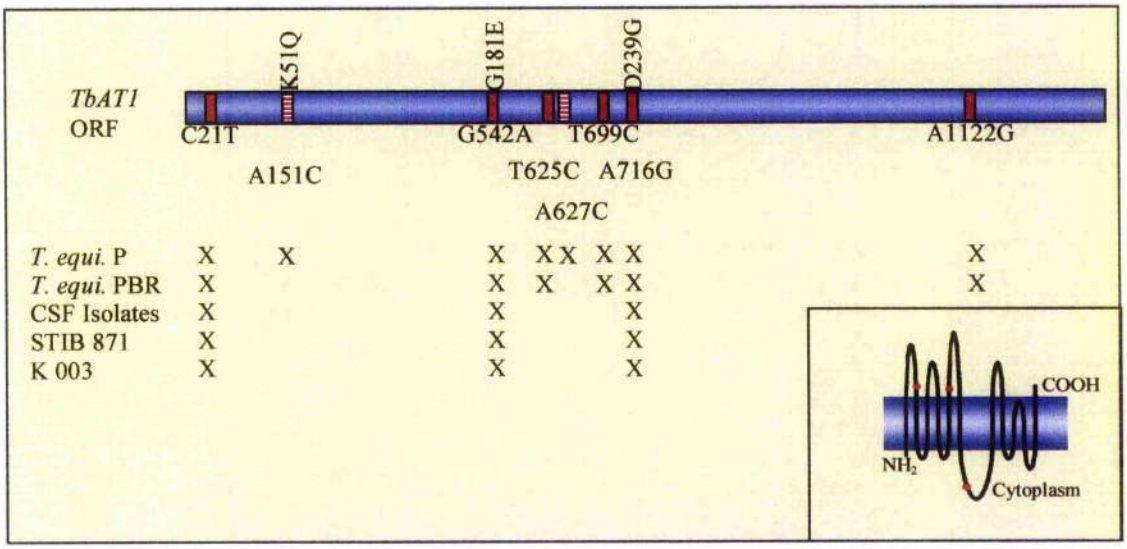


Figure 5.12. Position of mutations in *T. equiperdum TbAT1*.
The blue bar represents the *TbAT1* ORF as described in *T. brucei* 927. Mutations for which *T. equiperdum* appears to be heterozygous are shaded red and white, those that were present in all clones are in block red colour. The positions of changes in amino acid sequence between *T. equiperdum* and *T. brucei* are represented as red dots on the cartoon of predicted protein structure (insert). *T. equiperdum*, STIB 871, K 003 and the CSF isolates all have identical mutations at the positions indicated by a cross.

The amino acid change K51Q is predicted to occur on the first extracellular loop, and represents a change from a positively charged hydrophilic residue to a neutral polar residue. G181E is predicted to occur on the extracellular face of the transporter, and represents a change to a charged residue with a bulky side chain. D239G is predicted to occur on the large intracellular loop, and represents a change to a residue with a similar polar character, but carrying a less bulky side chain (Figure 5.12).

Further to the mutations in *TbAT1* illustrated above, the CSF isolates, STIB 871 and K003 also share one more mutation among themselves and a further five point mutations with STIB 777R (see Chapter I). Why there should be such conserved mutation 'hotspots' among isolates differing in species and location is subject to speculation.

The sequencing results indicate that *TbAT1* in *T. equiperdum* P exists as two alleles. The drug resistant *T. equiperdum* PBR cell line however, only appears to have the 'a' allele, with an adenosine at positions 151 and 627. This apparent loss of heterozygosity may account for the lack of P2 transcript and hence the loss of P2 function in *T. equiperdum* PBR. If the 'a' allele

in the P cell line, as sequenced in clones P6, P8 and P9, were to be transcriptionally silent or produce a non-functional transporter, drug resistance could then have come about by a loss of heterozygosity in the PBR line. However, the 'a' allele contains a sequence that is identical, at postions 151 and 627 of the ORF, to *TbAT1* from both *T. brucei* 427 and 927 wild type strains, which have fully functional P2 transport systems and a typical sensitivity to drugs. This would suggest that these mutations are unlikely to be instrumental in bringing about drug resistance.

Another possibility is that a silent copy of *TbAT1* has been created due to changes up or downstream of the open reading frame that regulate the expression of the 'a' allele. In this model, a mutation occurring at one allele (the 'a' allele) might silence that gene and then loss of heterozygosity would lead to silencing of the *TbAT1* locus. In the PBR cell line, it would appear that the active 'c' allele has been selected against, and a subsequent loss of heterozygosity has resulted in a loss of expression of the *TbAT1* gene. A loss of expression of *TbAT1* has been identified previously in *T. b. gambiense* 386 mr, another drug resistant cell line that has an unchanged *TbAT1* ORF from its parent cell line *T. b. gambiense* 386 (R. Burchmore, personal communication). Loss of heterozygosity may come about through the replacement of the active allele with the inactive one, or through the loss of one copy of the gene.

A loss of expression of *TbAT1* would be expected to effectively eliminate all P2 transport function in a cell line. However, a P2–like, adenine sensitive adenosine transport component was identified in the resistant cell line (Barrett et al. 1995) which had led to the original hypothesis that drug resistance had arisen from a change in affinity of the transporter for the substrate. Berenil, a commonly used diamidine drug is taken up solely through the P2 transporter (H. de Koning, unpublished data). Uptake assays in which berenil uptake is lost in *T. equiperdum* PBR (Figure 5.6) help verify a loss of P2 transport that a loss of expression suggests.

Blocking the uptake of adenosine with inosine and subsequently adenine shows that there still is an adenine sensitive adenosine transport component in the *T. equiperdum* PBR cell line (Figure 5.8). This was also identifiable in the *ΔTbat1* knock out cell line that has been confirmed to have no P2 transport activity (Figure 5.11). The adenosine uptake could be due

to the presence of an as yet unidentified adenosine transporter on the cell surface, one which could also interact with arsenical trypanocides. The TbNBT1 hypoxanthine transporter was identified as a possible candidate, but ruled out, as blocking its activity with an excess of hypoxanthine had no effect on the remaining adenosine uptake. This additional, low affinity adenosine uptake component could correspond to the third adenosine uptake component (adenine sensitive, berenil insensitive) previously reported in *T. evansi* (Ross & Barns 1996).

The loss of P2 function has been related to resistance to the melaminophenyl arsenicals and the diamidine classes of trypanocidal compounds. Table 5.5, below, shows the *in vitro* susceptibility to berenil and cymelarsan of *T. equiperdum* P and PBR lines, and the *ΔTbat1* cell line and its wild type parent.

Drug	IC_{50}		Resistance Factor	IC_{50}		Resistance Factor
	T. equiperdum P	*T. equiperdum* PBR		*TbAT1*	*ΔTbat1*	
Berenil (μg/ml)	0.0556	1.75	31.5	0.124	2.32	19
Cymelarsan (μg/ml)	0.005	0.02	4	0.0061	0.0124	2

Table 5.5. Resistance of *T. equiperdum* P and PBR and *TbAT1* and *ΔTbat1* cell lines to berenil and cymelarsan.

Resistance to berenil in both *T. equiperdum* PBR and *ΔTbat1* is high, 31.5-fold and 19-fold respectively. As berenil has been shown to access the trypanosome cell solely through the P2 transporter, the absence of this transporter in these cell lines would induce a high resistance factor. The relatively low resistance factor (4 and 2-fold) that both show to cymelarsan was unexpected. Cell lines that have been selected under laboratory conditions for resistance to arsenicals typically show very high levels of resistance, for example *T. b. brucei* 247 mr has a resistance factor of greater that 130-fold as compared to its parent cell line (Scott et al. 1996).

The results of the toxicity tests show that the loss of P2 alone does not account for these high resistance levels. Arsenical compounds could have another route of entry into the cells, and a

loss of this transport activity could lead to a higher level of resistance. Alternatively, the high levels of resistance seen in laboratory derived strains could be due to a series of changes in the target cell, brought about by the practice of constant selection under sub-curative doses of arsenical. The loss of function of P2 however, appears to be a conserved feature of drug resistance in many cell lines, and under drug pressure, could be one of those most readily induced.

The two- or four-fold difference in susceptibility to arsenicals that the loss of P2 brings about becomes very important when considering the pharmacokinetics of arsenicals in the CNS of infected patients. Arsenicals are present in the CNS at levels very close to the IC_{50} value of the drug. Even a two-fold loss in susceptibility could result in drug tolerant cells that could survive treatment within the CNS and cause relapse.

There remain many other genes that could potentially code for membrane bound nutrient transporters, including nucleoside and nucleobase transporters. The substrate recognition motifs for these transporters remain to be described, but they could possibly account for the adenine sensitive adenosine uptake seen in these P2 deficient cell lines.

Chapter VI

Development of a Rapid Fluorescence-Based Test for Drug Resistance in *Trypanosoma brucei*

6.1 Introduction

The current resurgence of Human African Trypanosomiasis (HAT), coupled with an increased incidence of drug resistance, is of great concern to health officials in affected areas (Barrett 1999). Relapse rates of 20 to 30% have been reported for treatment with melarsoprol, the principal drug of use against late stage infection (Brun et al. 2001). The only available alternative to melarsoprol treatment in arsenical-refractory infections is Difluoromethylornithine (DFMO). However, despite its efficacy against *T. b. gambiense* infection, DFMO is restricted in use due to high costs of administration and a limited supply (Etchegory et al. 2001). A quick, simple and sensitive clinical test capable of identifying drug resistant strains would allow health workers to decide on which course of treatment is most appropriate for each patient without having to wait for clinical relapse prior to making a choice.

The work presented here demonstrates a simple and effective assay that can distinguish between laboratory derived strains of parasite susceptible or resistant to treatment with commonly used trypanocidal compounds. The test has been developed using dicationic compounds that fluoresce when associated with DNA. These compounds also carry the recognition motif for the P2 purine transporter (Figure 6.1), the loss of which is closely associated with resistance to arsenical and some diamidine trypanocides (Barrett & Fairlamb 1999). It was anticipated that these compounds would be carried into the cell via the P2 transporter and the level of fluorescence achieved in live cells over a short period of incubation could distinguish between strains of parasite that are susceptible or resistant to treatment with commonly used trypanocidal compounds. This test may form the basis of an assay capable of detecting drug resistant trypanosome infections in both humans and cattle in the field.

6.1.1 Fluorescent Aromatic Dications

The aromatic dicationic compound pentamidine (Figure 6.1) has been used for over 60 years as a treatment for HAT (Wang 1995), being effective against early stage trypanosomiasis, when the parasites are in the bloodstream. It cannot cross the blood brain barrier in sufficient

quantities to clear trypanosomes that have established within the central nervous system, although it has been proposed to have some effect in the 'early late stage' of the disease (Doua et al. 1996). Pentamidine can also produce a range of side effects such as hypotension and renal and hepatic toxicity, and is inactive when administered orally due to poor gastrointestinal absorption.

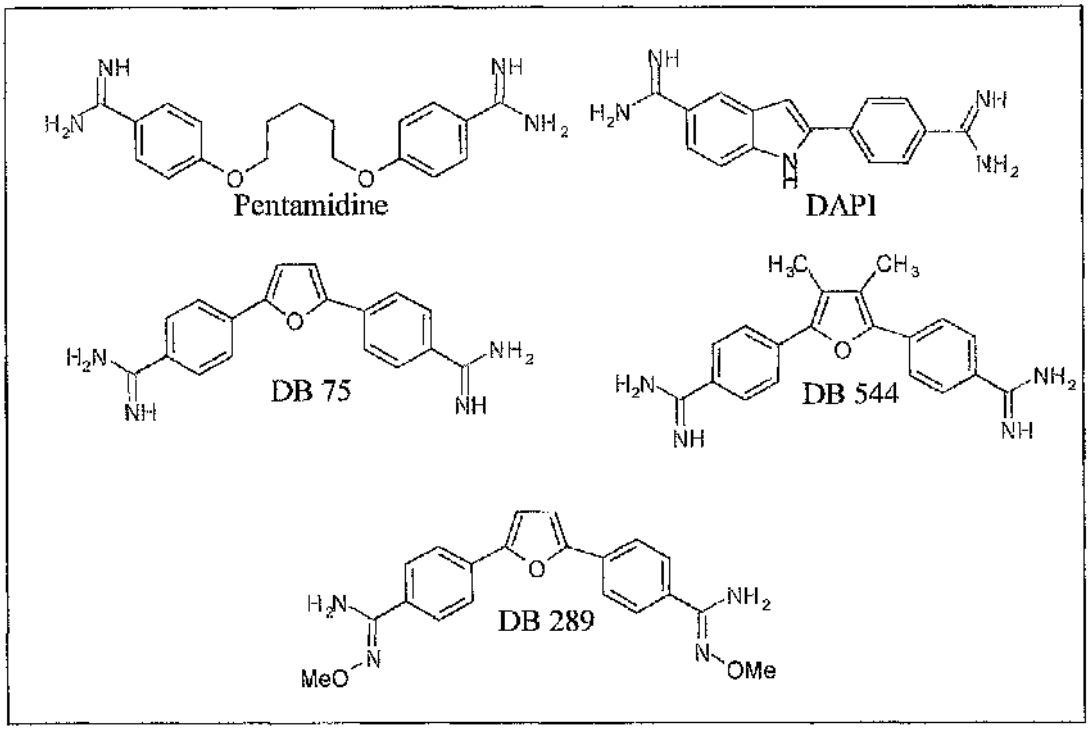


Figure 6.1. Chemical structures of pentamidine and analogues.
P2 recognition motifs are shown in red.

Despite these drawbacks, interest in pentamidine has increased due to its use as a secondary drug against AIDS related *Pneumocystis carinii* pneumonia (Smith & Gazzard 1991). Analogues have been developed in an effort to reduce undesirable side effects and to broaden the range of pathogens that can be treated.

A diphenylfuran, 2,5-bis (4-amidinophenyl) furan (DB 75, Figure 6.1) was found to be effective against many microbial pathogens when administered intravenously. When administered orally, however, DB 75 was only able to clear the intestinal parasite *Cryptosporidium parvum* (R. Tidwell, personal communication) among the pathogens tested. DB 75, like pentamidine, was found to be poorly absorbed across the gastrointestinal

epithelium. Further studies with analogues resulted in the synthesis of a range of aromatic diamidinoximes and other pro-drugs, which can be readily absorbed across the gastro-intestinal epithelium and are metabolised by the liver to their respective active dications.

DB 75 has already been shown to be active against trypanosomatids when administered parenterally (Das & Boykin 1977; Steck et al. 1981; Steck et al. 1982), but to only have very limited activity when administered orally. Further studies have shown that the dimethoxime of DB 75, 2,5-bis(4-amidinophenyl)furan bis-D-methylamidoximine (DB 289, Figure 6.1), is converted on absorption (probably by the liver) to DB 75. DB 289 is effective on oral administration against different animal models of early stage trypanosomiasis, has successfully completed phase I clinical trials, and is entering phase IIA trials in Angola and DRC (Legros et al. 2002). DB 289 and DB 75 are still, however, unable to clear infections with CNS involvement as, like other diamidines, they are unable to cross the blood brain barrier in sufficient quantities to reach trypanocidal levels.

The therapeutic diamidines in Figure 6.1 have two features in common which have been exploited in the development of this assay. These are their ability to interact with the P2 transporter and to fluoresce when bound to DNA. The ability of pentamidine and other diamidines to interact with the P2 transporter is due to the presence of the P2 recognition motif (de Koning 2001a) in the form of an amidine group at either end of the molecule. An ability to bind to DNA is common to many dicationic compounds and may be responsible, at least in part, for the antimicrobial action of these compounds (Wang 1995). Some of the novel dicationic compounds also fluoresce when bound to DNA, which makes their presence in cells, bound to the nucleus and the kinetoplast, easy to detect (Figure 6.2). This property is widely exploited in the case of 4',6-Diamidino-2-phenylindole (DAPI, a fluorescent diamidine, Figure 6.1) which is used as a standard DNA stain for many cell types.

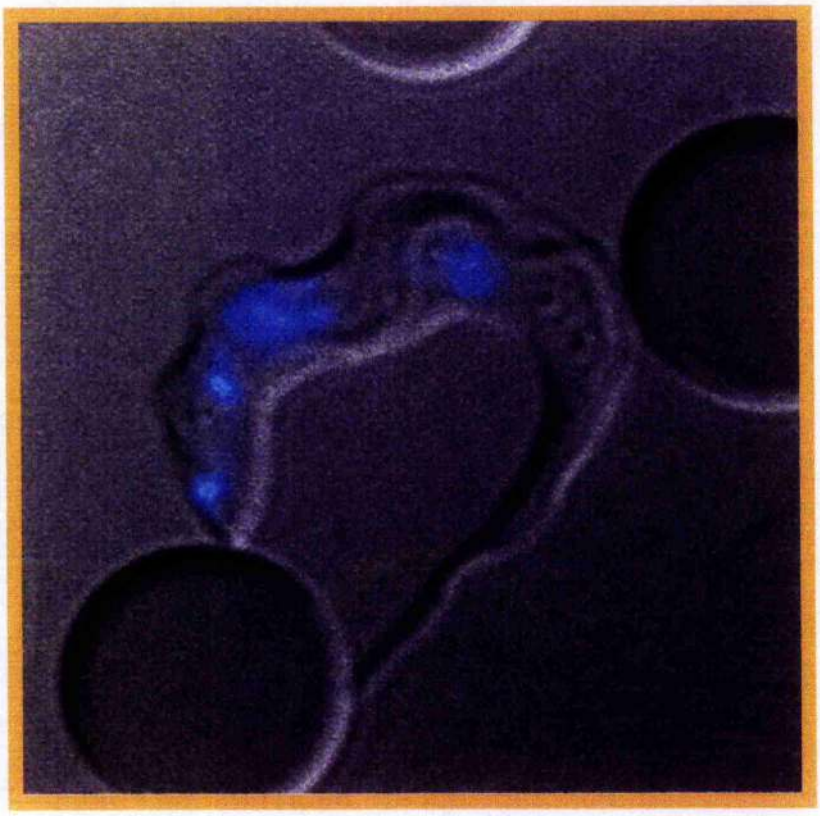


Figure 6.2. Live bloodstream form trypanosome stained brightly with DB 544.
Fluorescent compound associates with the DNA in the nucleus and the kinetoplast. This cell contained paired nuclei and kinetoplasts since it was in the process of division, prior to cytokinesis. Two flagella are also visible.

6.1.2 The Assay

A recent study from north western Uganda (Matovu et al. 2001a) has shown that *T. b. gambiense* isolated from patients were less susceptible to melarsoprol *in vitro* than parasites previously isolated in other foci in West Africa (minimum inhibitory concentrations of 0.009 to 0.072 μg/ml as opposed to 0.001 to 0.018 μg/ml). Within the study area, however, trypanosomes isolated from patients not responding to arsenical treatment are similar in sensitivity to arsenicals as those isolated from patients that responded to the drug.

It is possible that the overall slight decrease in susceptibility to arsenicals, coupled to host-related factors (e.g. permeability of extravascular sites to arsenicals), are responsible for the increased incidence of treatment failure in the study area. This seems more likely still when

taking into account that the quantity of melarsoprol that accumulates in the CSF is close to the normal effective concentration of the drug against wild-type parasites, (Keiser & Burri 2000; Keiser et al. 2000b). Data is not available for other sites, but failure to accumulate drug to trypanocidal levels in given extravascular localities would lead to relapse. Matovu and coworkers were also able to show some correlation between mutations in *TbAT1* (and presumably the loss of normal P2 transport function) and arsenical resistance.

The situation in the field regarding loss of P2 function and drug resistance remains ambiguous. However, the loss of P2 function is associated with arsenical resistance in all laboratory derived lines studied to date (R. Burchmore, personal communication), and is associated with a two-fold increase in resistance to arsenicals. Therefore a simple assay for P2 function, which could be used in a field situation, may be able to give an indication of resistance. This would allow health workers to prioritise patients with possible arsenical-refractory infections for treatment with DFMO. Such a test may be possible if the compounds described above were to gain entry into the trypanosome solely or predominantly via the P2 transporter. Their exclusion from the cell (indicated by the lack of fluorescence) would indicate a loss of transporter function and an increased resistance to arsenical treatment in that cell line.

A number of fluorescent diamidines were used to test whether they could be used in such a test. Compounds included DAPI, and the compounds DB 75 (2,5-bis 4-amidinophenyl furan) and DB 544 (2,5-bis (4-amidinophenyl) 2,3-bis methylfuran), two dicationic compounds known to fluoresce strongly when associated with DNA in the parasite, Figure 6.1.

6.1.3 Cell Lines Tested

6.1.3.1 *TbAT1/ΔTbat1*

The *ΔTbat1* (P2) null mutant clone was constructed from *T. b. brucei* strain MiTat 1.2 (BS221) (*TbAT1*) cloned cell line, (Enock Matovu, unpublished data). The P2 knockout cells show a 2 to 2.5-fold higher resistance to arsenical compounds and to pentamidine, and 18.6-fold higher resistance to diminazene aceturate, the active component of berenil. These results were derived by Alamar Blue assay and are shown in Table 6.1.

Trypanocidal Compound	*TbAT1* IC_{50} (μg/ml)	*ΔTbat1* IC_{50} (μg/ml)	Resistance factor
Melarsen Oxide	0.0037	0.0107	2.89
Cymelarsan	0.0061	0.0124	2.02
Diminazene	0.124	2.32	18.68
Pentamidine	0.005	0.012	2.4

Table 6.1. Sensitivity of *TbAT1* and *ΔTbat1* cell lines.
Cell lines were tested against arsenicals and diamidines.

6.1.3.2 *T. equiperdum* P/PBR

T. equiperdum P and berenil resistant *T. equiperdum* PBR. *T. equiperdum* PBR was selected *in vivo* for berenil resistance for which it is 36 times less sensitive. The PBR cell line is also 4-fold less sensitive to arsenicals, as shown in Table 6.2, (Zhang et al. 1992). Recent experiments (reported in Chapter V) have shown PBR to have an identical *TbAT1* open reading frame to the P strain. The absence of RNA detectable by Northern blot suggests that the deficiency in P2 transport in this cell line may be due to a lack of transcription of the gene (reported in Chapter V).

Trypanocidal compound	*In vivo* activity CD_{100} (mg/kg)		Resistance factor	*In vitro* activity MEC_{100} (μg/ml)		Resistance factor
	P	PBR		P	PBR	
Diminazene	18	>89	>5	0.0556	1.78	32
Cymelarsan	0.5	2	4	0.005	0.02	4

Table 6.2. Sensitivity of P and PBR cell lines, from Zhang, Z. Q., 1992.
CD_{100} = Curative dose in 100% of infected mice.
MEC_{100} = Minimum effective concentration killing 100% of trypanosomes in 24 hours of exposure.

6.1.3.3 *T. b. brucei* 247/247 mr

The arsenical resistant *T. b. brucei* 247 mr cell line was derived from the *T. b. brucei* 247 cell line. 247 mr was selected *in vivo* to increasing doses of cymelarsan. The resistant cell line is 130-fold less sensitive to cymelarsan, (Scott et al. 1996), 16-fold less sensitive to berenil and over 63-fold less sensitive to pentamidine (Alan Scott, Ph.D. Thesis, University of Glasgow) *in vivo* (Table 6.3). The 247 mr cell line has been shown to lack the *TbAT1* open reading frame

altogether, resulting in a 'natural knockout' of P2 transport (R. Burchmore, personal communication).

Trypanocidal compound	Minimum curative dose *in vivo*, (mg/kg)		Resistance factor
	247	247 mr	
Cymelarsan	0.3	>40	>130
Diminazene	2	32	16
Pentamidine	1.6	>100	>63

Table 6.3. Sensitivity of 247/247 mr cell lines.

6.1.3.4 *T. b. gambiense* 386/386 mr

The arsenical resistant *T. b. gambiense* 386 mr cell line was derived from the *T. b. gambiense* 386 cell line. The resistant cell line was also selected *in vivo* with increasing concentrations of cymelarsan. 386 mr is 20-fold less sensitive to cymelarsan treatment, Table 6.4, (Scott et al. 1996). Data for diamidines is not available. As with PBR, 386 mr has a full *TbAT1* open reading frame, but lacks detectable transcript (R. Burchmore, personal communication).

Trypanocidal compound	Minimum curative dose *in vivo*, (mg/kg)		Resistance factor
	386	386 mr	
Cymelarsan	2	>40	>20

Table 6.4. Sensitivity of 386/386 mr cell lines.
From Scott, A. G., 1996.

6.2 Results

6.2.1 Interaction of Aromatic Diamidines with Pentamidine Transporters

Pentamidine is known to enter trypanosomes through three separate transport systems (de Koning 2001b). P2 transports pentamidine with a K_m of 0.26 μM and a V_{max} of 0.068 pmol/10^7 cells/s. Two further pentamidine transporters have been identified in the trypanosome, the high affinity pentamidine transporter, HAPT1 (K_m = 0.036 μM, V_{max} = 0.0044 pmol/10^7 cells/s), and the low affinity pentamidine transporter, LAPT1 (K_m = 56.2 μM, V_{max} = 0.85 pmol/10^7 cells/s). HAPT1 has a high affinity, but only a limited capacity for

transporting pentamidine, which is reflected in its low V_{max} value. This means, that at physiologically significant concentrations, P2 and LAPT1 (with a low affinity but high capacity for transport) are the most important transporters with respect to the transport of pentamidine. Figure 1.10 shows the contribution that each transporter makes to uptake at any given pentamidine concentration.

Should the fluorescent analogues also be able to enter the cell via HAPT1 and LAPT1, their ability to indicate the absence of P2 transport function would be compromised. It was therefore necessary to determine the ability of each diamidine to act as a substrate for each transporter.

Pentamidine, DAPI and the compounds DB 75 and DB 544 have previously been screened for their ability to inhibit transport of radiolabelled substrates of these three transporters. Affinity for P2 was measured by their ability to inhibit the uptake of adenosine, and inhibition of pentamidine uptake was used to measure affinity for HAPT1 and LAPT1 (Table 6.5).

Compound	K_i (μM)		
	P2	HAPT1	LAPT1
DB 75	2.3	71.2*	No inhibition at 100 μM*
DB 544	1.99	15.3*	No inhibition at 100 μM*
DAPI	0.68*	26.6*	13.6*
Pentamidine	0.26*	0.0364*	56.7*

Table 6.5. K_i values of DB 75, DB 544 and DAPI for trypanosome pentamidine transporters.
* Results H. de Koning, unpublished data.

DB 75 and DB 544 have a high affinity for P2 but show no affinity for LAPT1 and limited affinity for HAPT1. DAPI also has a high affinity for P2. This compound however also has a relatively high affinity for LAPT1, Table 6.5 (H. de Koning, unpublished data). Further studies on the inhibition of adenosine transport via the P2 transporter using the oil-stop technique showed DB 75 and DB 544 have K_i values of 2.3 μM and 1.99 μM respectively (Figures 6.3 and 6.4).

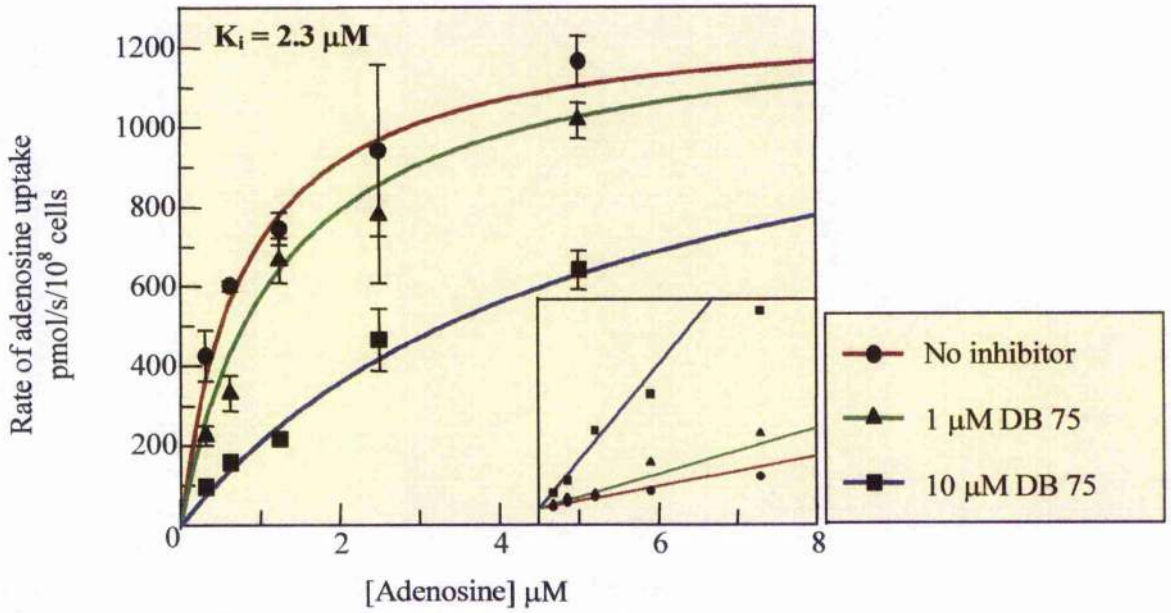


Figure 6.3. Inhibition of adenosine uptake by DB 75.
n = 3.

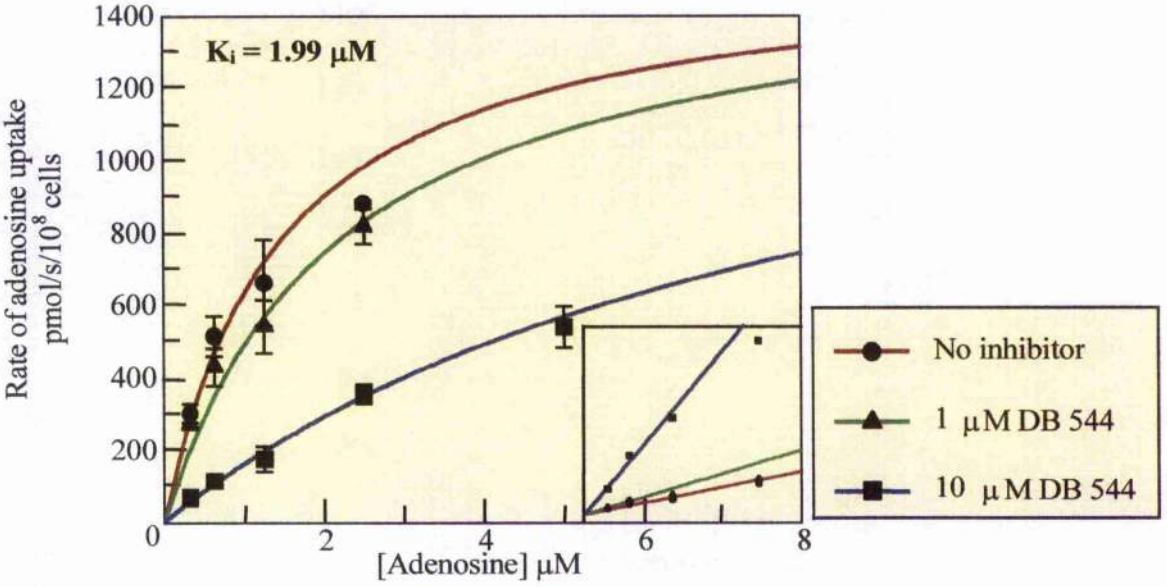


Figure 6.4. Inhibtion of adenosine uptake by DB 544.
n = 3.

The above results suggest that of all three pentamidine transporters, DAPI, DB 75 and DB 544 have the highest affinity for, and are likely to enter the cell mostly via P2. DB 75 and DB 544 appear to have no interaction with the low affinity, high capacity transporter LAPT1. Their

moderate affinity for HAPT1 could contribute to their uptake, however this transporter is very low capacity and as such would be expected to contribute very little to their internalisation at the concentrations used in this assay. DAPI has moderate affinities for both HAPT1 and LAPT1, which could compromise its ability to be useful in this assay.

6.2.2 Development of Fluorescence

6.2.2.1 DAPI

Fluorescence developed in *TbAT1* and *ΔTbat1* cells in the presence of DAPI as shown in Table 6.6.

Incubation time (min)	1 μM DAPI		10 μM DAPI	
	TbAT1	*ΔTbat1*	*TbAT1*	*ΔTbat1*
0	+	+	+	+
5	+	+	++	++
10	++	++	++	++

Table 6.6. Development of fluorescence in the presence of DAPI.
+ : visible fluorescence
++ : strong fluorescence

Fluorescence developed at the same rate in cells that had a fully functional P2 transporter and in those lacking P2. This may reflect the transport of DAPI by the pentamidine transporters, HAPT1 and LAPT1 for which it has moderate affinities, or other modes of uptake.

The failure of DAPI to differentiate between sensitive and resistant cell types led to this compound being dropped as a potential agent in the development of a drug resistance test.

6.2.2.2 DB 75

In order to optimise the concentration of DB 75 to be used in the assay, the development of fluorescence in the presence of 1 μM, 10 μM or 100 μM DB 75 was followed. The results are shown in Table 6.7.

Incubation time (min)	1 μM		10 μM		100 μM	
	TbAT1	*ΔTbat1*	*TbAT1*	*ΔTbat1*	*TbAT1*	*ΔTbat1*
0	-	-	+	-	+	-
5	-	-	+	-	+	-
10	+	-	+	-	+	+
15	+	-	+	-	+	+
20	+	+	+	+	+	+

Table 6.7. Development of fluorescence in the presence of 1, 10 or 100 μM DB 75.
+ : fluorescence
- : no fluorescence

Fluorescence developed at different rates in wild type and knockout cell lines in the presence of 1 μM, 10 μM and 100 μM DB 75. At 1 μM, fluorescence developed more slowly in the resistant cell line than in the sensitive cell line. The 10 μM concentration, however, gave a clearer and longer window between the development of fluorescence in the different cell lines (20 minutes for fluorescence to emerge in *ΔTbat1* lines compared with instantaneous appearance of fluorescence in wild type). A concentration of 100 μM DB 75 gave a shorter time difference in the development of fluorescence between the sensitive and resistant cell lines, and also had very high levels of background fluorescence, making it more difficult to distinguish fluorescing cells. A concentration of 10 μM was used for all subsequent experiments

The development of fluorescence in the presence of 10 μM DB 75 was followed over 10 minutes in all the cell lines described. The results are shown in Table 6.8.

Incubation time (min)	*TbAT1*	*ΔTbat1*	P	PBR	247	247 mr	386	386 mr
0	+	-	+	-	-	-	-	-
1	+	-	+	-	+/- *	-	-	-
2	+	-	+	-	+/- *	-	+	-
5	+	-	+	-	+/- *	-	+	-
10	+	-	+	-	+	-	+	-

Table 6.8. Development of fluorescence in the presence of 10 μM DB 75.
*Between 50 and 90 % of cells fluoresced in these samples. This is addressed in the text and appears to relate to the presence of stumpy forms.
+ : fluorescence
- : no fluorescence

None of the resistant cell lines, all of which lack P2 activity, developed fluorescence over the 10 minute time period. There was, however, a notable variation in the timing of fluorescence in the sensitive cell lines. The *TbAT1* and the *T. equiperdum* P cell lines both developed fluorescence immediately. The drug sensitive 247 and 386 cell lines took longer, developing fluorescence after 1 or 2 minutes incubation respectively.

The difference in the development of fluorescence in sensitive cell lines will be an important variable to note when developing this test for use in the field. The assay will have to allow long enough to account for these strain differences and reduce the possibility of false negatives. However, the duration of incubation must discount the possibility of fluorescence developing in resistant cell lines creating a falsely positive result. It will be important to assess a large number of cell lines to identify a reliable window allowing accurate diagnosis of P2 status and resistance. Based on the above data however, a 2-5 minute incubation time would be appropriate for identifying resistant and sensitive cell lines.

The observation that not all of the cells from the drug sensitive 247 cell line fluoresced, even though this was a clonal cell line, was also seen in subsequent experiments with the drug sensitive 386 cells, which are also clonal. Closer examination of blood smears revealed that, in murine infections, both 247 and 386 undergo cyclical parasitaemias (unlike *T. brucei* 427 and *T. equiperdum* derived lines), which result in fluctuating ratios of short stumpy cells (ss) to long slender cells (ls). Morphological examination of wet smears found that the ss cells, which are differentiated for survival in the tsetse fly, only began to develop fluorescence after 20

minutes of incubation, whereas the ls cells, the proliferative bloodstream form, developed fluorescence quickly.

T. brucei 427 ss forms have been shown to have down regulated P2 transport activity (H de Koning, unpublished data). This is perhaps because this transport function is not important in the subsequent procyclic form, the stage present in the midgut of the Tsetse fly. Studies with procyclic form 386 and 386 mr cells, showed that these cell types did not develop fluorescence at all over a 20 minute incubation, (Table 6.9), indicating that these lines may not express a functional P2 transporter

Incubation time (min)	Long slender cells		Short stumpy cells		Procyclic cells	
	247	386	247	386	386	386 mr
0	-	-	-	-	-	-
1	+	-	-	-	-	-
2	+	+	-	-	-	-
5	+	+	-	-	-	-
10	+	+	-	-	-	-
20	+	+	+	-	-	-

Table 6.9. Development of fluorescence in different life cycle stages and mixed ls and ss bloodstream infections with DB 75.
+ : fluorescence
- : no fluorescence

6.2.2.3 DB 544

DB 75 is currently undergoing trials for FDA approval of its use against *Pneumocystis carinii* pneumonia. For this reason the decision was made to test a very similar, fluorescent diamidine, DB 544, rather than introduce potential novel uses of DB 75 that might, in some way, add complexity to the decision process.

The development of fluorescence in the presence of 10 μM DB 544 was measured in all the cell lines described. The results are shown below (Table 6.10).

Incubation time (min)	*TbAT1*	*ΔTbat1*	P	PBR	247	247 mr	386	386 mr
0	+	-	+	-	-	-	-	-
1	+	-	+	-	+	-	+	-
2	+	-	+	-	+	-	+	-
5	+	-	+	-	+	+	+	-
10	+	-	+	-	+	+	+	+

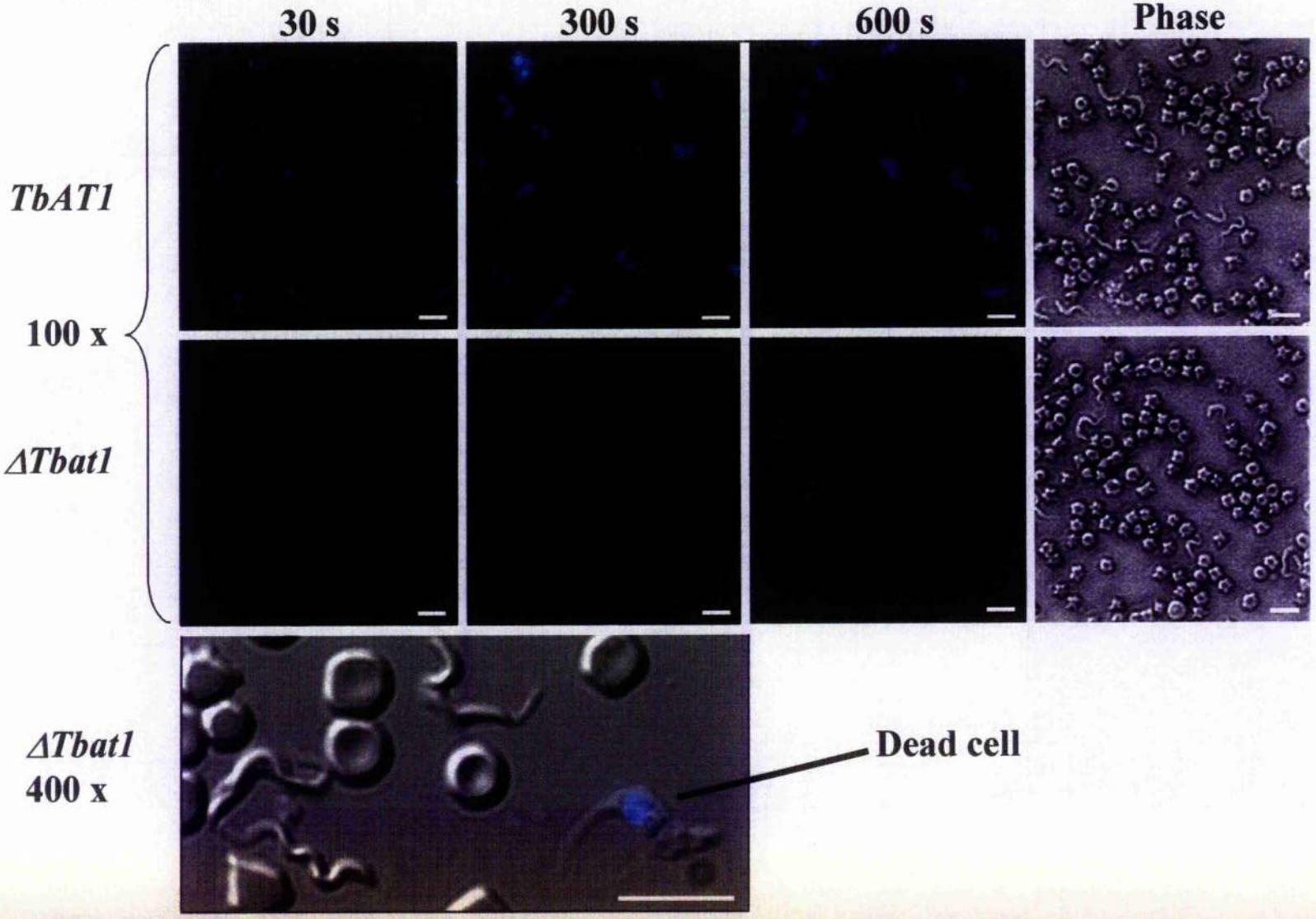
Table 6.10. Development of fluorescence in the presence of 10 μM DB 544.
+ : fluorescence
- : no fluorescence

Fluorescence develops more quickly in the 247 mr and 386 mr cell lines in the presence of DB544 than in the presence of DB 75. There is still a time difference between the development of fluorescence in the sensitive and resistant cell lines, but it is not as long as that for DB 75. Because of this, DB 75 would be a better choice for using in a field application.

As in DB 75, short stumpy forms of 247 and 386 did not develop fluorescence in the presence of DB 544 over 10 minutes.

A time course for the development of fluorescence in the presence of DB 544 in *TbAT1* and *ΔTbat1* cells was acquired. The results are shown in Figure 6.5. Fluorescence develops in the sensitive cells almost immediately, but is only just visible on close examination in resistant cells at ten minutes. This leaves a window of at least ten minutes in which it is possible to differentiate between arsenical sensitive and resistant cell lines by the presence or absence of fluorescence. Isolated points of fluorescence were visible among the *ΔTbat1* cells at all time points, but examination under phase contrast revealed this to be confined to dead cells.

Figure 6.5. Time course for development of fluorescence in *TbAT1* and *ΔTbat1* cells in the presence of DB 544.
Scale bars are 10 μm.



6.2.3 Blocking the Development of Fluorescence with Adenosine and Pentamidine

Fluorescence is dependent on the uptake of compound through a functional P2 transporter. The delayed fluorescence in the sensitive strains demonstrates that there are further routes into the cells for the compounds, the most likely candidates being the pentamidine transporters. To further clarify the situation we attempted to block the development of DB 75 fluorescence by pre-incubating cells for 30 seconds with an excess of inhibitory substrate before adding the fluorescent compounds. P2 uptake was challenged with 1 mM adenosine, and all pentamidine transporter uptake, including that by P2, was challenged with 1 mM pentamidine. The results are shown in Table 6.11.

Incubation time (min)	Incubation with 1 mM adenosine		Incubation with 1 mM Pentamidine	
	TbAT1	*ΔTbat1*	*TbAT1*	*ΔTbat1*
0	-	-	-	-
5	-	-	-	-
10	+	-	-	-
15	++	-	-	-

Table 6.11. Inhibition of DB 75 fluorescence with adenosine and pentamidine.
++ : fluorescence
+ : weak fluorescence
- : no fluorescence

1 mM pentamidine is able to completely block the development of fluorescence in the sensitive and resistant cell lines tested when pre-incubated with the cells for 30 seconds. A pre-incubation with 1 mM adenosine in *TbAT1* cells results in a time lag before fluorescence is seen, which is similar to that seen in *ΔTbat1* cells without the pre-incubation (Table 6.7).

These data give further credence to the theory that the delayed development of fluorescence in cell lines lacking P2 transport activity could be due to uptake of the fluorescent compound via the pentamidine transporters, HAPT1 and LAPT1. However, since pentamidine is also a dicationic molecule that interacts with DNA, this large excess of pentamidine may also be able to block fluorescence by competing with the fluorescent compound for binding sites in the DNA.

6.2.4 Trypanotoxicity

The fluorescent diamidines were observed to be trypanotoxic. DB 75, DB 544, DAPI and diminazene aceturate (berenil) were all tested for toxicity against *T. b. brucei* 427 and *TbAT1* and *ΔTbat1* cells *in vitro*. The results are in Table 6.12.

Compound	IC_{50} (μM)			
	T. brucei 427	*TbAT1*	*ΔTbat1*	Resistance factor
DB 75	0.033	0.0454	5.282	116.19
DB 544	0.033	0.0344	1.77	51.53
DAPI	0.38	0.477	1.9	3.98
Diminazene	0.14	0.124	2.32	18.6

Table 6.12. Trypanotoxicity of pentamidine analogues, DAPI and diminazene aceturate.
Resistance factor is derived from the difference in sensitivity between the *TbAT1* and *ΔTbat1* cell lines.

The very high resistance factor between *TbAT1* and *ΔTbat1* cells for DB 544 and DB 75 could reflect their very high specificity for uptake by P2, whereas DAPI, which has moderate affinities for LAPT1 and HAPT1 has a low resistance factor. The high resistance factors of DB 544 and DB 75, as compared to that for diminazene aceturate, is however inconsistent with separate observations that diminazene aceturate is only internalised through P2 (H. de Koning, unpublished data). An uptake assay for the *TbAT1* and *ΔTbat1* cell lines for the uptake of radiolabelled diminazene aceturate over time shows a lack of uptake in the *ΔTbat1* cells that lack a functional P2 transporter (Figure 6.6).

As the DB compounds have been shown to be internalised in cells lacking P2, perhaps through the LAPT1 and HAPT1 transporters, in a three day toxicity assay their resistance factor would be expected to be lower than that for diminazene aceturate, which is apparently excluded by lack of transport. An as yet undetermined route of entry for diminazene aceturate, or an effect of the drug on the cell surface could explain the apparent inconsistencies. For example, diminazene aceturate may adhere to plasma proteins and be internalised by endocytosis. Alternatively it could act on some part of the trypanosome cell surface as pentamidine does to Ca 2+ pumps in the plasma membrane (Benaim et al. 1993). This would serve to decrease the resistance factor, even though all active transport has been stopped.

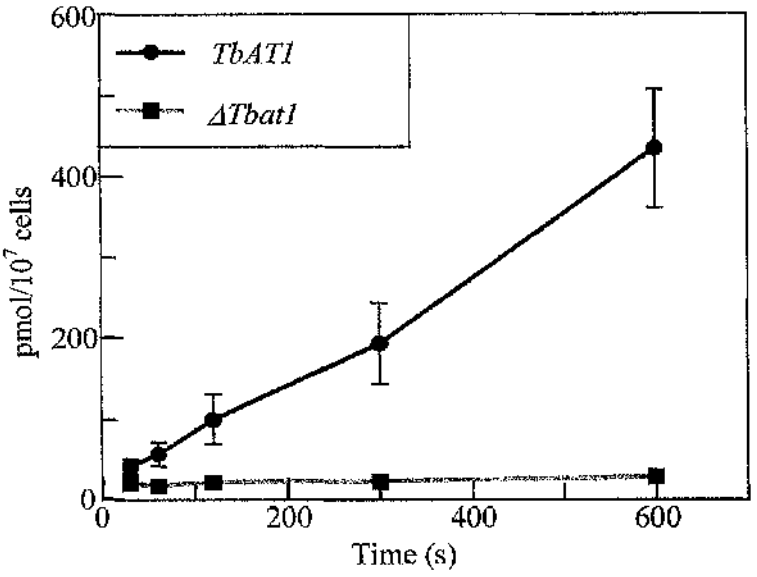


Figure 6.6. Uptake of berenil in *TbAT1* and *ΔTbat1*.
Uptake of 20 μM berenil over time. Bars indicate standard deviations, n = 3.

6.3 Discussion

Drug resistance, particularly to melarsoprol, appears to be of increasing incidence in sleeping sickness-affected Africa. Diagnostic tests for resistance are critical in enabling decisions on patient management with respect to treatment regimens to be made early. This chapter outlines a test that diagnoses drug resistance in four out of four parasite lines tested.

All laboratory-derived arsenical-resistant isolates that have been studied have altered P2 activity (Burchmore and Barrett, unpublished). Moreover, if, as may be indicated in recent research (Matovu et al. 2001a; Matovu et al. 2001b), the loss of P2 is among the first events leading to drug resistance in the field, the test described above could provide health workers with a useful diagnostic tool. As a test for P2 function, and therefore resistance to arsenical and diamidine drugs, the rapid fluorescence technique described here is much more accessible than other techniques that have been suggested previously.

One previously suggested assay for P2 function involves the identification of point mutations in the *TbAT1* gene by PCR followed by digestion of the product with *Sfa NI* (Mäser et al. 1999). This is based on the observation that an arsenical-resistant cell line selected in the

laboratory had 10 point mutations, one of which deleted a *Sfa NI* digestion site and one of which created one. A similar pattern of point mutations has been identified in field isolates of *T.b. gambiense* taken from relapse patients in north-western Uganda and northern Angola and in a *T. b. rhodesiense* strain from south-eastern Uganda (Matovu et al. 2001b). *Sfa NI* digested PCR products, run on a gel, exhibit distinct banding patterns depending on which *Sfa NI* restriction site is present.

This test confers no advantage over the fluorescence technique as far as the requirement of sophisticated equipment is concerned. In this assay a PCR machine and electrophoresis equipment would be needed along with cold storage for molecular reagents. Also, loss of function of P2 is not always associated with changes to *Sfa NI* digestion sites. 247 mr, for example, lacks *TbAT1* altogether, and 386 mr and PBR have intact *TbAT1* genes with no changes compared to their wild type parent strains that have full P2 activity. Loss of transcription of the gene appears to underlie the loss of function phenotype in these cases. A test based on changes in this single *TbAT1* restriction site would therefore not be able to firmly diagnose drug resistance in these cell lines. It is likely that many different mutations, leading to altered P2 function, could emerge in the field as well as the laboratory. The fluorescence test measures the loss of function phenotype regardless of particular genotype.

Another option for a drug sensitivity assay is the culture of cells from infected patients *in vitro* followed by an *in vitro* drug sensitivity test. However, *T. b. gambiense*, isolated from the field, is very difficult to grow *in vitro*. Cells must first be established in an experimental animal and then adapted for *in vitro* culture. This is a time consuming practice taking weeks to derive reliably growing stocks, which is prone to failure (Matovu et al. 2001a). The rapid fluorescence test has the advantage of not relying on any specific change to the *TbAT1* gene, the measure of this test being for functional expression of P2, regardless of what events may have led to the loss. This technique is also very sensitive, requiring only very few trypanosomes for diagnosis. Thus, in terms of speed and sensitivity, it is far superior to any other tests that have been proposed to date.

6.3.1 Development of the Rapid Fluorescence Test for Arsenical Resistance for use in the Field

In spite of the advantages of the rapid fluorescence test for arsenical resistance there are several challenges to be overcome in the development of this test for the field, not least of which is access to a fluorescence microscope. Only larger field hospitals may have the resources necessary to work with a fluorescence microscope. This means that samples will have to be collected and then transported to another site for diagnosis. It may be possible to develop a technique for fixing the trypanosomes after exposure to the fluorescent diamidine (live cells are required to show active transport of the diamidine). Slides could then be transported and assessed for fluorescence. While a fixing procedure followed by transport would reduce the speed of diagnosis, this assay would remain faster than the alternatives outlined above.

The manner of fixation of cells exposed to fluorescent marker is vitally important. On fixation, cells, regardless of their origin, have a tendency to become 'leaky'. This would allow fluorescent compound to flood into, and associate with the DNA of all cells, sensitive or resistant, resulting in false diagnosis of resistant cells as sensitive. This could be overcome by adding a large excess of fixative to the cells to greatly dilute the fluorescent diamidine or by adding an anionic 'sponge' to the buffer, after a period of incubation, to soak up excess diamidine, e.g. herring sperm DNA or poly-aspartic acid. Both of these methods could be used in conjunction with centrifuging the cells through an oil layer after incubation to separate them from the free diamidine in solution.

Moreover, recent advances in microscopy technology should make it possible to design and construct field microscopes suitable for fluorescence work. It may be possible to use a car headlamp or even sunlight as a light source with filters to produce the correct wavelength, or a light emitting diode (LED) of the appropriate wavelength, eliminating the need to transport samples to another location. Recent advances in LED technology have led to the development and marketing of LEDs that emit light of wavelengths appropriate for the test developed here. This would mean samples could be assessed *in situ* and the appropriate treatment could begin at once.

Using a fluorescence-adapted field microscope in conjunction with a specially developed slide designed to capture trypanosomes from a blood sample could also resolve another potential problem, namely that of low parasitaemias which are often presented in *T. b. gambiense* infections. The low parasitaemias presented means that mini-anion exchange chromatography, as used in routine diagnosis, will also be required for isolating trypanosomes for the test. As an alternative, it may be possible to capture trypanosomes on a slide coated with an anionic polymer. Since trypanosomes are positively charged at pH 8 they will stick to the negatively charged polymer while erythrocytes (negatively charged at pH 8) will not. The blood cells would then be washed off and the trypanosomes incubated with fluorescent compound (Figure 6.7).

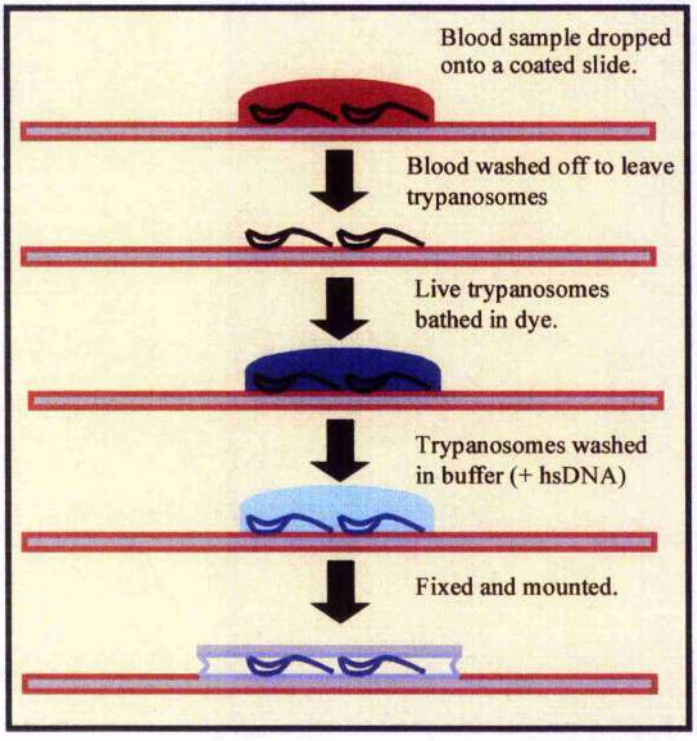


Figure 6.7. Potential set up for the Rapid Fluorescence Test for Drug Resistance.
hsDNA = Herring Sperm DNA, could be added at this point in order to act as an anionic sponge, to capture excess DB 75.

The whole preparation could then be viewed wet, or fixed and mounted to be viewed at another time. Once assessed for fluorescence, this procedure has the additional advantage in that the wet or fixed cells can also be viewed under phase contrast. An experienced microscopist would at this stage be able to remove a further problem by identifying what proportion of the cells, if any, are short stumpy cell forms, and therefore would not fluoresce, possibly due to a lack P2 transport activity, eliminating the possibility of a falsely positive result for drug resistance.

On a final note, this test, and the equipment described, has the potential to be adapted for the diagnosis of drug resistance in many organisms as well as in cancer cells.

Chapter VII

General Discussion

This project aimed to investigate the potential of the P2 purine transporter as a 'gateway' for drug entry and its role in resistance to trypanocidal drugs. The first aim was pursued by connecting the primary substrate recognition motif of the P2 transporter, $H_2N–C(R_1)=N(R_2)$, to a moiety which was potentially toxic to the trypanosomes. It was anticipated that the recognition motif would allow the toxic compound to be internalised specifically by the trypanosomal cells, reducing the potential toxicity for the mammalian host.

Most rational drug design relies upon designing inhibitors of parasite-specific targets, which differ sufficiently from mammalian analogues to confer specificity. The majority of these potential drug targets are, however, inside the parasite cell, and this necessitates their entry into the parasite cell in order for them to hit their appropriate target. Their entry could be achieved by designing a lipophilic compound that can cross any cell membrane including that of the mammalian host. There is, however, a drawback to this approach in that, although toxic or inhibitory compounds can be designed to a target specific to the parasite cell, a compound that has access to any cell increases the risk of toxicity to host cells.

The approach to drug design used in this project involved targeting toxic substances specifically to the trypanosomes by exploiting the action of a parasite membrane transporter with a high affinity for a particular substrate, and a recognition motif for that substrate unlike any in mammalian cells. The P2 transporter is ideal for this approach to drug design (Hasne & Barrett 2000) due to its high affinity for substrates carrying the recognition motif, the lack of any similar motif on mammalian purine transporters, its natural substrates being at very low concentrations within the bloodstream of the host and its likely ability to internalise substrate against a concentration gradient (de Koning & Jarvis 1999).

The toxic moieties used included polyamine analogues, nitrofurans and nitroimidazoles and HIV gp120 binding mimetics, as well as compounds selected from a database. The P2 recognition motif was added in the form of either a melamine or a benzamidine group, or, for all of the database compounds except Distamycin A, was present in the form of a guandine group.

The results from the database compounds indicated that the guanidine group could not act as a substrate for P2. The only compound in this group to show any interaction with the P2 transporter was Distamycin A, which carries an amidine group. The HIV gp120 binding mimetics 509 and 510 also carry a guanidine motif, and these have up to 24-fold less affinity for the P2 transporter than their benzamidine analogues, again reflecting the loss of affinity that guanidine residues confer (Table 4.2). In these compounds it is possible that the loss of affinity is attributed to the spatial configuration of the primary P2 recognition motif with respect to the π orbitals of the benzene ring.

High affinity binding to the P2 transporter requires an aromatic system on the substrate (de Koning & Jarvis 1999). It is proposed that the π-orbitals of the substrate stack with the π-orbitals of an aromatic residue within the substrate binding site to increase the binding energy of the interaction and stabilise the bond. From results reported in this thesis, it also appears that the P2 transporter requires the primary recognition motif and the aromatic system to be in a planar configuration for optimal binding, as is reflected in the higher affinities for P2 in the planar benzamidine and melamine compounds as compared to the non-planar guanidine compounds.

All of the compounds that carried either a melamine or a benzamidine recognition motif were able to inhibit the uptake of adenosine via the P2 transporter. There are general patterns in their ability to inhibit P2 transport, with respect to their structures, as outlined below.

The polyamine analogues demonstrate an increasing affinity for P2, as measured by apparent K_i values, with an increase in the length of their central chain. This may reflect the amount of time a longer compound takes to traverse the cell membrane and for the transporter to reconfigure itself to receive another substrate molecule. The longer each inhibitor molecule is associated with the transporter and is blocking the uptake of the labelled substrate, the lower the K_i will appear. This means that a compound with a low affinity that travels across the membrane slowly may appear to have the same K_i as a compound that has a high affinity but a faster turnover. In order to determine whether this is the case with these polyamine analogues, labelled compounds would be required to determine transport kinetics.

Affinity for P2 is consistently greater in the majority of melamine compounds bearing one monomethylated or dimethylated melamine amine on their transport units than for their unmethylated analogues. It is possible that the methyl substitution of one amine group might increase the electron density in the compound's π-system, which in turn could increase the contribution of π-stacking interactions to the binding of the compound, whilst leaving one amine group open as the primary recognition motif for the transporter. The addition of any more methyl groups disrupts the P2 recognition motif and decreases affinity.

The benzamidine compounds in this study show greater affinities for P2 than most of the melamine bearing compounds. However, *in vitro* toxicity experiments for these compounds against *TbAT1* and the P2 transport deficient *ΔTbat1* cell lines reveal no difference in toxicity, suggesting that the P2 transporter is not critical in the delivery of these compounds. There is a possibility that any difference in toxicity due to P2-specific uptake has been masked due to a diffusion component.

Further studies using analogous benzamidine- and melamine-bearing compounds would further clarify the situation regarding a possible structural preference for the benzamidine group over the melamine group in P2 interactions. If this were to be the case, then future compounds would benefit from carrying a benzamidine in place of a melamine.

Some compounds showed very high toxicities towards trypanosomes *in vitro* but most demonstrated moderate toxicities, and a few, most notably the database compounds and the nitroimidazoles, appeared to lack any toxicity *in vitro*.

Although many of the database compounds have therapeutic properties or uses, they were chosen on the basis of the presence of the P2 recognition motif, and they lack any trypanocidal activity *in vitro*. The results also show that nitrofurans are generally much more toxic to trypanosome cells than the nitroimidazoles. The activities of these compounds are related to the reduction potential of their nitro groups. As nitrofurans are more easily reduced than nitroimidazoles, their different redox potentials could explain the differences in their respective toxicities, although this could also increase host toxicity.

The melamine-bearing compounds showed increasing toxicities corresponding to increasing methylation of the melamine amines (Chapters III and IV). None of the compounds, methylated or unmethylated, showed any difference in toxicity towards a *ΔTbat1* (P2 knockout) cell line, which would indicate that P2 is not important in the uptake of these compounds or in their toxic effects. *In vivo*, however, the compound 744 was able to clear or delay the development of infections whereas its monomethylated analogue, 745, had no influence on parasitaemia (Table 4.4). This lack of *in vivo* activity in the methylated compound 745 indicates that methylation, by decreasing toxicity, has opposite effects *in vivo* and *in vitro*, which could be related to either an influence on uptake or interactions with intracellular targets.

744 represents a potential lead compound in the search for new trypanocides. Successful early and late stage animal trials have been conducted (Reto Brun, Swiss Tropical Institute, personal communication), and further work is needed on this compound to assess its mode of action and its usefulness as a trypanocidal drug *in vivo*.

Although 744 is clearly very active *in vivo* at therapeutic levels, there remains concern about its mode of action, in that the reduction of the nitrofuran group by mammalian cells could result in damage to host cells. One way of potentially avoiding this host toxicity would be to target the toxic moiety even more specifically to the trypanosome cell. There is evidence from this study that benzamidines interact with P2 with greater affinity than melamine-bearing compounds. In order to clarify the situation, it would be beneficial to synthesise test compounds bearing the nitrofuran group and a benzamidine group. If a benzamidine compound is scavenged more effectively by the trypanosome cells it could be administered in smaller doses, and would be less able to access mammalian cells, reducing the likelihood of host toxicity.

In order to investigate the respective uptake properties of benzamidine and melamine compounds with respect to the P2 transporter, we also require analogous compounds to be synthesised carrying a label of some kind, for example radioactivity or fluorescence. Furthermore, genes that could code for membrane bound nutrient transporters are being identified through the trypanosome genome project. The substrate recognition motifs for these

transporters remain to be described, but they could offer access routes, which could be exploited as parasite-specific gateways into the cell.

While the development of novel reagents that might enter trypanosomes via the P2 transporter is critical given the crisis in HAT chemotherapy, the concern that the loss of this transporter could lead to drug resistance is very real. Therefore a more detailed understanding of the role of the loss of P2 in drug resistance was required. The role of the P2 transporter in the development of Berenil resistance in a *T. equiperdum* strain which had been reported to have a P2 transport component with greatly reduced affinity for adenosine was investigated. The gene encoding P2 transport activity, *TbAT1*, was analysed in this cell line, *T. equiperdum* PBR, and its Berenil-susceptible parent cell line *T. equiperdum* P. *TbAT1* from both cell lines carried six nucleotide point mutations resulting in two changes at the amino acid level as compared to *TbAT1* described in *T. brucei* 927. However, the sequencing results indicate that *TbAT1* in *T. equiperdum* P exists as two alleles, one of which has two further point mutations in the sequence ('c' allele). The *T. equiperdum* PBR cell line does not contain the 'c' allele, only having sequence corresponding to the first 'a' allele as found in *T. equiperdum* P.

The *T. equiperdum* PBR cell line lacks any *TbAT1* transcript detectable by Northern blot, which suggests that this loss of heterozygosity in *T. equiperdum* PBR has resulted in a lack of transcription of the gene. This may have come about due to changes up or downstream of the open reading frame, that regulate the expression of the 'a' allele. A subsequent loss of heterozygosity in the *T. equiperdum* PBR cell line has resulted in a *TbAT1* gene that is not expressed.

This loss of expression was expected to delete all adenine sensitive adenosine uptake from the *T. equiperdum* PBR cell line. However, a low affinity adenine sensitive adenosine uptake component was identified through inhibition experiments. The nature of this transport component was not identified, but it was also identified in a *ΔTbat1* cell line and probably equates to the third adenosine transporter described in *T. evansi* by Ross and Barns, 1996.

The loss of P2 function has been related to resistance to the melaminophenyl arsenicals and the diamidine classes of trypanocidal compounds. The *T. equiperdum* PBR cell line and a

ΔTbat1 cell line, both of which lack P2 transport activity, show high level resistance to Berenil, and low level (four-fold and two-fold respectively, Table 5.5) resistance to arsenicals. The loss of P2 function in these cell lines has additionally been shown to only give low level resistance to the melamine and benzamidine compounds in this study. It would appear that, although these compounds where designed for uptake via P2, they have other routes of entry, which reduces the likelihood of drug resistance developing rapidly through the loss of P2 function to these compounds designed with melamine and benzamidine motifs.

Nonetheless, the loss of function of P2, appears to be a conserved feature of drug resistance in many laboratory derived cell lines, and under drug pressure, could be one of those most readily induced. Additionally, the loss of P2 transport activity has been linked to the development of drug resistance in the field. Although it appears that high levels of resistance to arsenicals are brought about through different mechanisms than P2 loss, it is possible that a two-fold loss in sensitivity is enough to allow parasites in the CNS to survive treatment and reinvade other tissues.

A test for loss of function of P2 would be a valuable tool for both research into the prevalence of the loss of function of P2 in the field and as a diagnostic tool for clinicians. The rapid fluorescence test described here identifies the loss of function of P2, regardless of the molecular basis for that loss, in four out of four parasite lines tested. As a test for P2 function, and therefore resistance to arsenical and diamidine drugs, the rapid fluorescence technique as described here is much more accessible than other techniques that have been suggested previously.

In conclusion, new trypanocidal compounds have been synthesised, one of which shows promise as a lead compound for drug development. These compounds have been shown to interact with the trypanosome P2 transporter, but do not rely solely upon the presence of this transporter for uptake into trypanosome cells, reducing the likelihood of the development of resistance to these compounds through the loss of the transporter. As loss of P2 has been identified as contributing to drug resistance in both laboratory derived cell lines and field isolates, a quick and simple test has been developed that is capable of identifying cells lacking the P2 transporter, and are therefore tolerant to pharmacologically relevant levels of diamidine

and arsenical trypanocides. This simple test could be adapted for use in the field either for research purposes, or by clinical staff, with the aim of identifying patients with drug-resistant trypanosomiasis, and tailoring their treatment accordingly.

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Appendix I

CBSS

Hepes	25 mM
NaCl	120 mM
KCl	5.4 mM
CaCl	0.55 mM
$MgSO_4$	0.4 mM
Na_2HPO_4	5.6 mM
D-glucose	11.1 mM

Adjust to pH 7.4.

PSG

PS Buffer

Na_2HPO_4 (anhydrous)	13.48g
$NaH_2PO_4.2H_2O$	0.78g
NaCl	4.25g

Final volume of 1L in dd water at pH 8.

To 6 volumes PS add 4 volumes dd water with 1% glucose. Adjust to exactly pH 8.

Lysis Buffer

Tris-Cl pH 8	10 mM
EDTA	100 mM
Sarkosyl	1%
Proteinase K	100 µg/ml

TAE

Trizma base	4.84 g
EDTA	0.372 g

Made up to 1 L in dd water adjusted to pH 8 with glacial acetic acid.

Luria Bertani Medium (LB)

Bacto-tryptone	10 g
Bacto yeast extract	5 g
NaCl	10 g

Made up to 1 L, pH 7, sterilised by autoclaving.

20x SSC

Tri-sodium citrate	88.23 g
NaCl	175.32 g

PH 7-8, final volume 1 L.

Denhardt's Solution

Bovine Serum Albumin	2 g
Ficoll	400.2 g
Polyvinylpyrolidone	2 g

Final volume of 100 ml, stored at -20oC.

20x Northern Gel Buffer

Na_2HPO_4	0.36 M
NaH_2PO_4	0.04 M

Formaldehyde Gel-loading Buffer

Glycerol	50 %
EDTA pH 8	1 mM
Bromophenol Blue	0.25 %
Xylene cyanol FF	0.25 %

Blue Sample Loading Buffer

Bromophenol Blue	0.25%
Xylene cyanol FF	0.25%
Sucrose in water	40% (w/v)

Sometimes, if you pay real close attention to
the pebbles you find out about the ocean.