Amino Acid Transporters & Amino Acid Metabolism in *Trypanosoma brucei brucei*

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1 Author's Declaration

The results presented in this thesis are my own work, except where stated otherwise.

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2 Abstract

The development of new drugs against Human African Trypanosomiasis is much needed due to toxicity, efficacy and availability problems with current drug treatments for this resurgent parasitic disease. Delivery of drugs into cells is an important determinant of therapeutic efficacy of drugs. An effective means of selective drug delivery is to use plasma membrane transport systems to mediate the entry of drugs into the cell.

Some amino acid transporters fulfil the criteria needed for successful exploitation of nutrient transport systems for drug delivery. The *Trypanosoma brucei* genomic database was screened to identify the full gene repertoire of amino acid transporters. From this, candidate genes were selected and functional genetic approaches were employed to characterise candidate amino acid transporter genes. Further characterisation of TbAATP1, a RNAi cell line shown to be a transporter of small neutral amino acids (serine, glycine, cysteine, asparagine and alanine), showed a role in threonine uptake.

Amino acid analogues were tested for trypanocidal activity. Of the 96 tested, two (Azaserine and Levodopa) were investigated in more detail, paying special attention to the nature of their trypanocidal action and possible route of entry through an amino acid transporter. Azaserine showed a trypanostatic action as well multiple routes of entry into the protozoan interior (as shown by inhibition of glutamine, phenylalanine and tyrosine uptake). The trypanocidal Levodopa showed entry through a tyrosine specific transporter. However, it is possible that Levodopa's trypanocidal activity may not be as a result of the analogue itself, but secondary products of the analogue.

Amino acids are important for protozoa as energy sources as well as forming pools of soluble osmolites. Amino acid usage in trypanosomes was investigated. Upregulation of proline transport and catabolism in response to reduced glucose availability was exhibited by the genome strain of *T. brucei*. Moreover, this metabolic shift could be mimicked by addition of GlcNAc to the medium, which blocks the hexose transporter limiting glucose entry to the cell. Systems biology approaches were initiated to investigate the undergoing metabolic changes. More specifically, mass spectrometry methodologies were employed to investigate underlying metabolite changes in procyclic form trypanosomes grown in differing medium.

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6 Definitions

AATP	amino acid transporter
ADP	adenosine diphosphate
Ala	alanine
Arg	arginine
ASCT	acetate:succinate CoA transferase
Asn	asparagine
Asp	aspartic acid
ATP	adenosine triphosphate
BBB	blood brain barrier
BSF	bloodstream form
bp	base pair
ºC	degrees Celsius
CBSS	Carter's Balanced Salt Solution
cDNA	complementary DNA
CNS	central nervous system
CSF	cerebro-spinal fluid
Cys	cysteine
DFMO	difluoromethylornithine
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
2-DOG	2-deoxy-D-glucose
DON	6-diazo-5-oxo-l-norleucine
dsRNA	double stranded RNA
DTT	dithiothreitol
EDTA	ethylenediamine tretraacetic acid
EEG	electroencephalogram
ES	expression site
FAD	flavin adenine dinucleotide
FCS	foetal calf serum
g	gram
Gln	glutamine
Glu	glutamic acid
Gly	glycine
gRNA	guide RNA
h	hour
HAT	human African trypanosomiasis
His	histidine
IAEA	International Atomic Energy Agency
Iso	isoleucine
IPTG	isopropylthio-ß-D-galactoside
kb	kilobase
kg	kilogram
Km	Michaelis constant
LB	Luria-Bertani medium
Leu	leucine
Lys	lysine
M	molar
Mb	megabase
Met	methionine
µg	microgram
mg	milligram

min	minutes
mM	millimolar
µM	micromolar
µl	microlitre
ml	millilitre
mRNA	messenger RNA
MSF	Médecins sans Frontières
MTS	mitochondrial targeting sequence
NAD+	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
nM	nanomolar
nm	nanometre
ORF	open reading frame
PARP	procyclic acidic repetitive protein
PCF	procyclic form
PCR	polymerase chain reaction
PBS	phosphate buffered saline
Phe	phenylalanine
Pro	proline
RNA	ribonucleic acid
RNAi	RNA interference
RNA pol	RNA polymerase
rpm	revolutions per minute
RT-PCR	reverse transcriptase PCR
secs	seconds
SEM	standard error of the mean
Ser	serine
SDM	semi-defined medium
SDS	sodium dodecylsulphate
SSC	saline sodium citrate
Taq	Thermus aquaticus
TbAATP	*Trypanosoma brucei* amino acid transporter
Thr	threonine
Tm	melting temperature
TM	transmembrane
Trp	tryptophan
Tyr	tyrosine
UV	ultraviolet
V	volt
Val	valine
Vmax	maximal velocity
VSG	variable surface glycoprotein
WHO	World Health Organisation
X-Gal	5-bromo-4-chloro-3-indol-ß-D-galactopyranoside
ZPFM	Zimmerman's post fusion media

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And finally, I would like to thank my family, especially my parents, whose love, belief and understanding have always and will always be there for me, wherever I am on this Earth.

In conformity and with thy free judgement, in whose hands I have placed thee, thou art confined by no bounds; and thou wilt fix limits of nature for thyself. I have placed thee at the centre of the world, that from there thou mayest more conveniently look around and see whatsoever is in the world. Neither heavenly nor earthly, neither mortal nor immortal have I made thee. Thou, like a judge appointed for being honourable, art the molder and maker of thyself; thou mayest sculpt thyself into whatever shape thou dost prefer. Thou canst grow downward into the lower natures which are brutes. Thou canst again grow upward from thy soul's reason into the higher natures which are divine.

- ***Giovanni Pico della Mirandola***
- ***"Oration on the Dignity of Man"***

1 Introduction

The underlying goal of this project is to assess the suitability of amino acid transporters to deliver trypanocidal compounds to the interior of the trypanosome, encompassing the investigations of selected aspects of amino acid usage (from transport capacity to metabolic usage). Molecular, biochemical, pharmacological and ultimately systems biology approaches were employed, specifically to: (1) characterise amino acid transporter genes by RNA interference; (2) test amino acid analogues for trypanocidal activity; (3) test the ability of GlcNAc to induce a metabolic shift from glucose to proline metabolism; (4) investigate changes to the trypanosome metabolome as a result of utilisation of different carbon sources.

1.1 The Disease

Parasitic protozoa infect hundreds of millions of people every year and are collectively some of the most important causes of human misery (Barrett *et al.*, 2003). Human African Trypanosomiasis (HAT), or sleeping sickness, is in resurgence (Moore & Richer, 2001). Devastating epidemics of HAT re-emerged in many sub-Saharan countries in the late 1990's (Stich *et al.*, 2003). The invariably fatal disease puts at risk 60 million of the 400 million people inhabiting 36 sub-Saharan African countries (Barrett, 1999). Of those at risk from the disease only a mere 4 million are under surveillance (Etchegorry *et al.*, 2001). The annually reported cases are therefore an underestimation of the real problem.

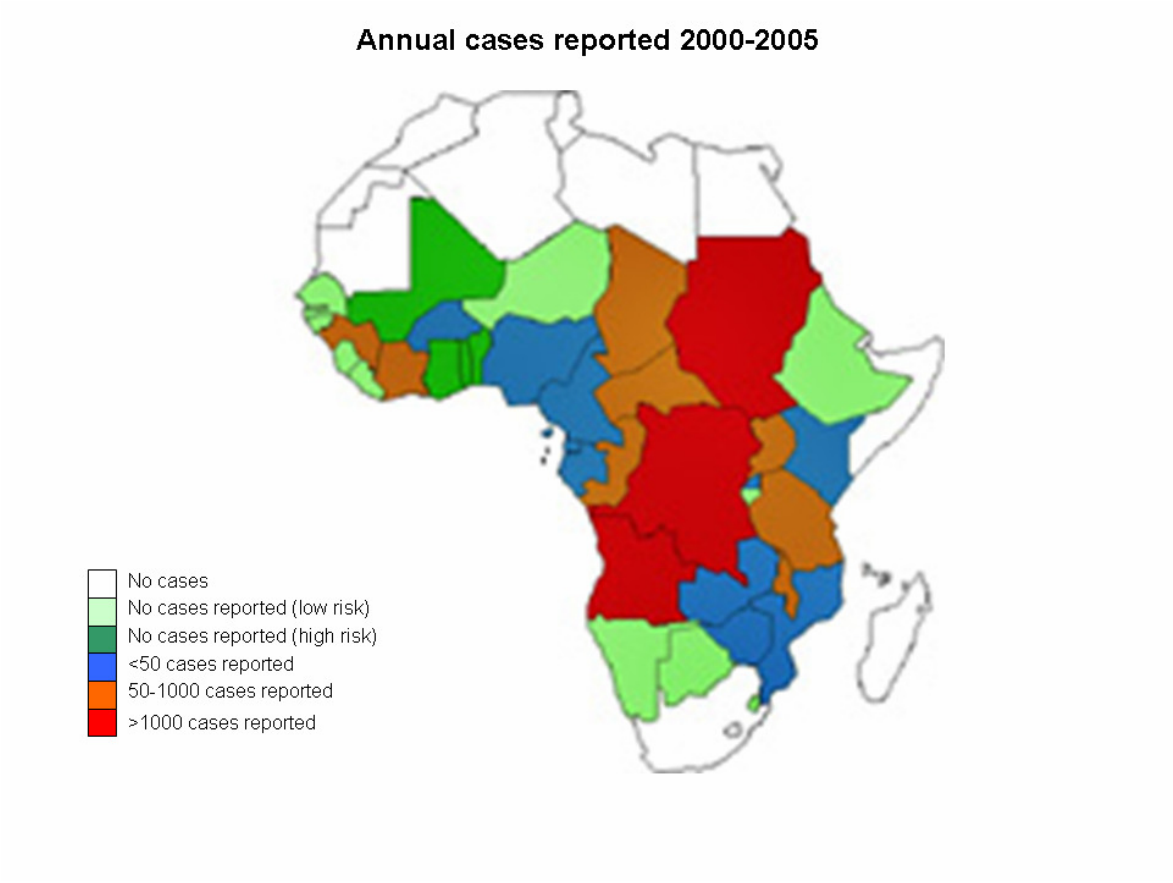


Figure 1-1: Human African trypanosomiasis endemicity in the WHO African Region. Number of reported cases from 2000-2005 (www.who.int).

The disease spreads in poor settings. Displacement of populations by war and poverty are important factors which lead to increased transmission. The disease develops in areas whose size can range from a village to entire regions, and within a given area, the intensity of disease can vary. There have been several human epidemics of HAT in Africa over the last century. The major one occurred between 1896 and 1906, and affected mostly Uganda and the Congo basin. This was followed by one in 1920 which struck a number of African countries. The disease did resurge in the 1970's leading to alarming epidemics in the 1990's. Recently in 2005, outbreaks were still reported in Angola, the Democratic Republic of Congo and Sudan. In the Central African Republic, Chad, Congo, Côte d'Ivoire, Guinea, Malawi, Uganda and United Republic of Tanzania the threat of HAT still remains an important public health problem, with 50-1000 new cases reported every year (www.who.int). Countries such as Burkina Faso, Cameroon, Equatorial Guinea, Gabon, Kenya, Mozambique, Nigeria, Rwanda, Zambia and Zimbabwe are reporting fewer than 50 new cases per year (Figure 1-1). In countries such as Benin, Botswana, Burundi, Ethiopia, Gambia, Ghana, Guinea Bissau, Liberia, Mali, Namibia, Niger, Senegal, Sierra Leone Swaziland and Togo transmission seems to have stopped and no new cases have been reported for several decades (www.who.int). However, any assessment of the current situation of sleeping sickness in Africa will always be, at best, an underestimate due to the fact that a lack of surveillance as well as clinical and diagnostic expertise does exist.

Trypanosoma species of the order Kinetoplastida are early branching eukaryotes and are a major public health problem across the new and old world. *Trypanosoma brucei* is the agent of two major afflictions affecting the African continent, sleeping sickness (human form) and nagana (animal form) (Lecordier *et al.*, 2005). Possibly no other disease, with the exception of Malaria, HIV and Tuberculosis, has a stranglehold on the development of a continent that trypanosomiasis has on Africa. The disease in domestic animals especially cattle, is also a major obstacle to the economic development of the region. Biting tsetse flies of the genus *Glossina* transmit the subspecies of the salivarian *Trypanosoma brucei* group across east, west and central Africa to both livestock and humans. The "Gambian" infection, caused by *Trypanosoma brucei gambiense*, which accounts for 90% of reported cases, is characterised by a long asymptomatic stage (2 or more years) that gives way to febrile illness followed by late stage chronic meningoencephalitis (Murray *et al.*, 2000). The "Rhodesian" infection caused by *Trypanosoma brucei rhodesiense* progresses more quickly (few weeks to 2 months). *Gambian* infection has a mainly human reservoir while *Rhodesian* infection is fuelled by a large animal reservoir.

During the first stage of HAT, after infection from the bite of an infected tsetse fly, parasites spread to the draining lymph node and bloodstream. General malaise, headache, fever, peripheral oedema and anaemia characterise the early stages, which may be accompanied by a series of other more problematic symptoms like myocarditis, pulmonary oedema, splenomegaly and hepatomegaly. These are problematic because these symptoms are not specifically diagnostic for African sleeping sickness. These symptoms are also associated with other diseases, frequently leading to misdiagnosis (Bogitsh *et al.*, 2005).

Second stage is more serious, with the parasites crossing the blood-brain barrier and invading the central nervous system. This invasion is accompanied by infiltration of lymphocytes. General malaise worsens, headaches become more severe and patient sleep pattern is altered as a result of alterations in brain function later escalating in to coma and eventually leading to death (Barrett *et al.*, 2003).

1.2 Vector Biology & Control

According to some authorities, trypanosomiasis has kept Africa 'green'. By allowing survival of the native fauna and preventing overstocking of fragile land with cattle, and hence preventing widespread erosion. Conversely, trypanosomiasis disrupts the development of sustainable mixed farming over large and potentially productive areas (Hursey, 2001). The ultimate result being agricultural impoverishment. A burden that accounts for a reduction in agricultural production, and further enhances the struggle of this developing continent.

The long history of trypanosomiasis control started at the turn of the 20th century with the identification of the infectious agents and their vectors (Evans, 1881; Dutton, 1902; Kleine, 1909 – as reviewed by Cox, 2004). The first stage of disease control must always be stopping transmission. This is even more apt for trypanosomiasis as clinical treatment has always been problematic (chapter 1.5). Different species of tsetse have different habitats, mainly found in vegetation by rivers and lakes, in gallery forests and in vast stretches of wooded savannah (www.who.int/mediacentre/factsheets/fs259/en/). Control of the tsetse fly vector was initially a large undertaking. Large areas of vegetation where the flies rested were cleared and the wild game that acted as an animal reservoir for the disease were shot (Schofield & Maudlin, 2001). Although proving effective, such methods are not employed today. Synthetic insecticides were the next major advent in disease control. During the 1940's large landmasses were sprayed with Dichloro-Diphenyl-Trichloroethane (DDT), pyrethroids, and other organochlorides (Vreysen, 2001; Vreysen *et al.*, 2000). Although

Glossina was once widespread, it is now limited to continental Africa south of the Tropic of Cancer (Bogitsh *et al.*, 2005). Tsetse transmitted trypanosomiasis affects a 9-million km^2 area in sub-Saharan Africa (Hursey, 2001). Vector life cycle is an important facet of transmission. Fertilised eggs, within the female, hatch at intervals into larvae (Bogitsh *et al.*, 2005). A single female tsetse will deposit up to 10 larvae at any one time, usually at 9 day intervals. The adult fly emerges after a month in gestation.

Tsetse control in Africa has become multi-faceted. Fly biology, behaviour and life cycle have allowed more methods of control. The problem of insecticide resistance faced by mosquito control has never been seen with tsetse flies, possibly due to slower reproduction time of the flies (female flies gestate once every 9 days). Some problems faced at the turn of the 21st century still remain, but an understanding of the seasonal movements of the flies to more humid areas during the dry seasons has allowed use of this natural limitation of tsetse movement to great advantage (Schofield & Maudlin, 2001). Ground and aerial spraying remain the first method of effective control. However, despite being effective, these processes are not only costly but also labour and management intensive. Continuous monitoring, sustainability and vigilance are serious requirements to prevent against re-invasion and re-infestation of cleared areas (Schofield & Maudlin, 2001). This sustainability relies on effective co-operation of concerned governments and the consolidating of peoples affected. International declarations, programme activities, mandates and campaign strategies are a step forward, but without the infrastructure needed to maintain such initiatives, Africa will always fall victim to trypanosomiasis.

The other form of control is on a much smaller scale and involves odour-baited traps and applying insecticides to the animal reservoir on which tsetse flies feed. Odour traps attract flies over a relatively small distance towards visual 'targets' (dark blue) treated with insecticides (Schofield & Maudlin, 2001). This local population control, together with cattle dipping helped reduce the vast amounts of insecticides used when spraying (Esterhuizen *et al.*, 2006; Torr *et al.*, 2007) and also meant that the control method was sustained all year round, which is an added advantage.

In recent years, control has taken a more molecular biology orientated approach, using molecular genetics and vector-parasite interactions to help curb the resurgence of the disease. These control methods are often seen as 'high-tech' especially when compared to the control methods already previously discussed. These high-tech methods employ detailed analysis of tsetse fly variation, population structure, evolutionary dynamics, behaviour, susceptibility, and ecology as the basis for control (Aksoy, 2003). The sterile

insect technique (SIT), where infertile flies are released into wild populations to compete with natural males, is a method that employs genetic population suppression with the ultimate result of eradication. An area-wide tsetse eradication project was initiated in Zanzibar in 1994, consisting of the release of gamma-sterilised males and resulting in the successful eradication of *Glossina austeni* from the island of Unguja (Vreysen *et al.*, 2000). The SIT has the advantage in that tsetse flies are slow to reproduce, meaning eradication is a distinct possibility. However, the large numbers of irradiated sterile males needed and the implementation of such a release method mean that it is not viewed as a favourable method of control (Rogers & Randolph, 2002).

All tsetse are potential vectors, and their presence thus presents a risk for parasite transmission (Fèvre *et al.*, 2006). With all species of *Glossina* able to carry the parasite, vector biology is becoming more important in terms of control. The focus in the past has been only to concentrate on the human-parasite interaction. Now, investigations are underway to identify factors that influence parasite transmission as a way to intervene at the level of the fly. The flies own innate immune system and trypanocidal midgut lectins are just two potential areas that could lead to fruitful knowledge. There is still a huge deficit in what is known on tsetse biology (Aksoy *et al.*, 2002). Information on the genome, proteins and metabolic pathways are needed to understand how the parasite infestation works, with the hope of ultimately bringing about new applications for vector control.

1.3 Life Cycle

Introduction of the infective stage of the parasite into the human host begins with the bite of an infected tsetse fly vector (Figure 1-2). Blood clotting is prevented by factors in the saliva of the insect (Bogitsh *et al.*, 2005). The metacyclic trypomastigote is a distinctive life cycle stage not only in terms of morphology but also in certain biochemical characteristics. Its morphology is blunt with a short free flagellum and the mitochondrion has few cristae and it has diminished classical electron transport complexes (Vickerman, 1985). Metacyclic trypanosomes transform to the long slender form, eventually finding their way into the cerebrospinal fluid (chronic form of the disease diagnosed by parasites found in lumbar punctures). The mammalian bloodstream form stage exhibits three distinct forms; the long slender form with a free flagellum extending from the undulating membrane, a short stumpy form and an intermediate form that is not that clearly defined (Vickerman, 1985).

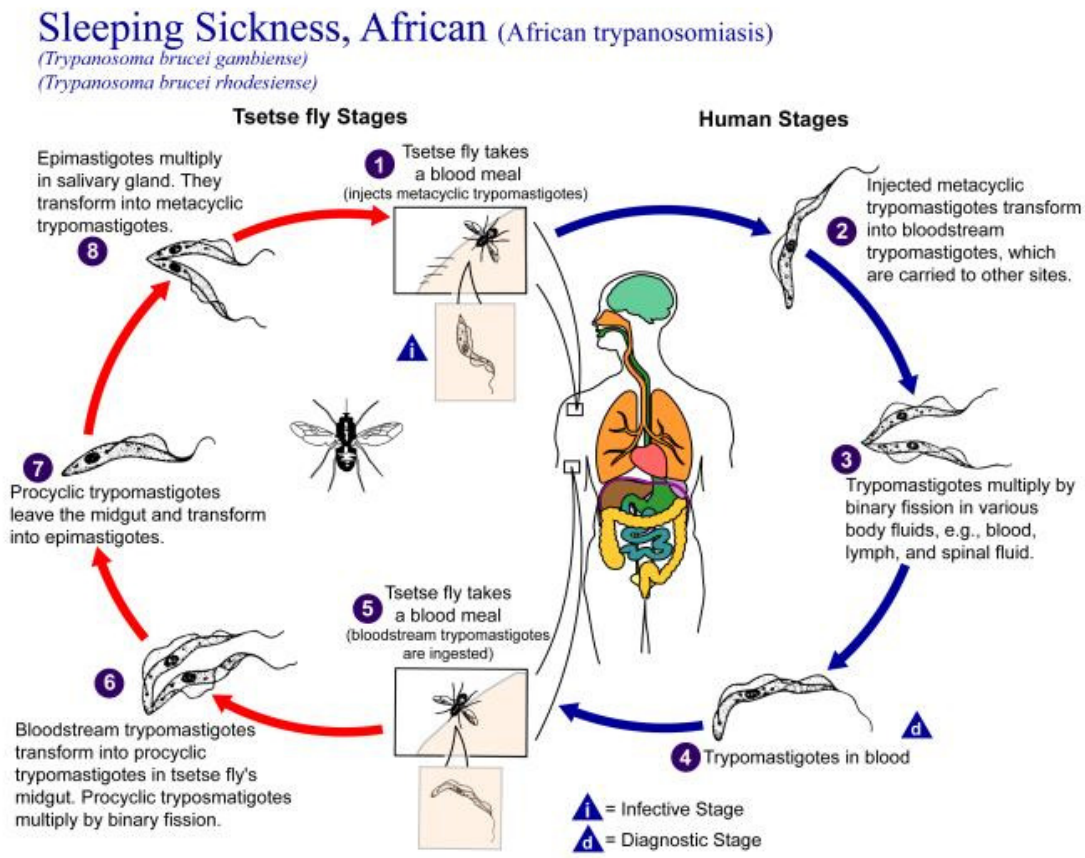


Figure 1-2: Life cycle of the African trypanosome in the human and tsetse fly.
Image credit: da Silva & Moser, Public Health Image Library.

Completion of the life cycle comes with ingestion of infected blood by the tsetse fly vector. The short stumpy form bloodstream trypomastigote is pre-adapted for life within the vector. Upon entering the midgut of the fly complete adaptation is rapid. Transformation to the procyclic insect form is characterised by the loss of the surface coat and antigenic identity. Procyclics invade the extraperitrophic spaces, migrating anteriorly towards the foregut where they transform once again to the epimastigote form trypomastigote. Migration continues through the oesophagus into the salivary gland of the fly. The final metamorphosis comes with the transformation of the epimastigote into the mammalian infective metacyclic form (Bogitsh *et al.*, 2005).

1.4 Control Strategies

Control of African trypanosomiasis is multifactorial and relies initially on identifying (usually by microscopic detection of parasites on a thick blood smear) the largely asymptomatic human reservoir (Murray *et al.*, 2000). Diagnosis forms one step in the

range of control strategies. The diagnosis of HAT is based upon a combination of clinical and investigative data (Kennedy, 2004). The non-specific nature of many of the clinical symptoms, together with the epidemiological context of geographical location shared with many other tropical infections means it is important to rule out other diseases such as malaria, tuberculosis, HIV and various other wormal infections. Differentiating between acute and chronic stages of the disease is the next important step once the disease has been confirmed. This is mainly due to the drug treatment regimes that differ for the different stages of the disease.

The definitive diagnosis of the *rhodesiense* form of the disease is made by identifying parasites in the peripheral blood using thin or thick films (Kennedy, 2006). For the *gambiense* form of the disease, serological methods are used due to the fact that parasitaemia is usually intermittent. The card agglutination trypanosomiasis test (CATT) is a quick and easy test for screening potential HAT cases and also allows the collection and storage of dried blood samples on filter-paper for future serological analysis (Magnus *et al.*, 1978; Truc *et al.*, 2002). Parasite identification, however, is crucial given the dangers associated with treatment (chapter 1.5).

CNS involvement with HAT is the key issue for diagnosis and subsequent therapeutics. Hence, a lumbar puncture for collection of cerebro-spinal fluid (CSF) is essential. The criterion for defining late-stage HAT as set by the World Health Organisation (WHO) is based on the presence of trypanosomes in the CSF and/or a CSF white blood cell count of >5/uL (WHO, 1983). However, the CSF white blood cell count cut-off varies for different countries (www.who.int). Other methods of detection exist for diagnosis of CNS involved HAT, which include PCR, raised CSF proteins, EEG abnormalities, and the presence of intrathecal IgM synthesis. However, such tests are not easily applicable in the field and as such are not convenient methods of diagnosis.

Vector control and chemotherapeutic regimes are present as control strategies. With no vaccine under development, the impetus is to improve existing strategies and formulate new drug regimes. Emphasis is on development of new orally effective trypanocides and vector control with new technology (Stich *et al.*, 2003). Halting the surgence of the disease is a realistic goal. This can only come from the co-operation of the international public health communities as well as the drug companies that once, and to some extent still do, view development of anti-trypanosomiasis drugs as not economically viable. Evidently, the emergence of public-private partnerships aimed at developing new drugs for various afflictions of the developing world have been exceptional (Barrett, 2006). Initiatives and

funding bodies like the Drugs for Neglected Diseases Initiative (DNDi), the Wellcome Trust, the Bill & Melinda Gates Foundation, Médecins Sans Frontières (MSF), Malteser, WHO/Special Programme for Research and Training in Tropical Diseases (WHO/TDR), and Caritas, to name just a few are providing not only the funds but also the infrastructure to combat HAT from the bench to the field. Moreover, the WHO surveillance and control programme collaborates with a number of international organizations, research institutions, development agencies, NGOs and private firms to achieve its objective of ensuring that a broader context of the disease, in terms of social and urban development, is met (http://www.who.int/mediacentre/factsheets/fs259/en/).

A network of field researchers, operational agents, epidemiologists, and scientists work under an umbrella created by the WHO in order to monitor drug resistance as well as finding solutions to the problems of treatment of trypanosomiasis (Etchegorry *et al.*, 2001); including the dissemination of information on current regimes, availability and affordability of current drugs, as well as the promotion of research on the causes of treatment failure (Legros *et al.*, 1999). Treatment failure is a big issue when combating sleeping sickness, especially *gambiense* sleeping sickness (Brun *et al.*, 2001). The fact that treatment relies on very few drugs means that resistance and relapse plays an important part in disease burden. Refractoriness and unresponsiveness to drug also fall under the term treatment failure. Reasons for this are not always parasite related, with host related factors (patient's metabolism resulting in altered pharmacokinetics) also a relevant cause.

Some initiatives are already in place (Stich *et al.*, 2003). The release of sterile tsetse flies has been proposed by the International Atomic Energy Agency (IAEA). The WHO in collaboration with MSF are encouraging drug companies to donate the vital drugs needed and commit themselves to continued production. In addition, funding from notable benefactors like the Bill & Melinda Gates Foundation has allowed progression of drug trials for novel drug candidates (Jannin & Cattand, 2004). Campaigns initiated to stimulate efforts in combating neglected diseases such as this perpetuate the false belief that persistence of disease is solely due to a lack of effective and affordable treatments by focussing on the absence of appropriate research and the unaffordability of current drugs (Anon, 2006). Access to medicines is as much a crucial issue as the development of new drugs, and only when infrastructures are put in place to aid support delivery of drugs to people most in need will the disease burden be lightened.

No vaccines exist against sleeping sickness, and the prospects of prophylactic immunisation are poor since the parasites change their surface coat periodically in a

process termed antigenic variation (Van der Ploeg *et al.*, 1992). Current drug regimes suffer many drawbacks. Some are decades old, toxic and would never achieve licensing today. This is the problem faced.

1.5 Chemotherapy

Only four drugs are licensed for treatment against African sleeping sickness: suramin (Germanin), pentamidine (Pentamidine isethionate), melarsoprol (Arsobal) and eflornithine (Ornidyl). Drug of choice depends on whether the parasite has established within the central nervous system. Suramin first came into use in 1922 and is a colourless polysulphonated symmetrical naphthalene derivative (Voogd *et al.*, 1993). Suramin covers treatment of early stage sleeping sickness, but is the drug of choice for *Rhodesian* infection. The drug's mode of action is obscure. The highly charged molecule accumulates in trypanosomes very slowly and is believed to be taken up via receptor-mediated endocytosis possibly bound to low density lipoproteins (Vansterkenburg *et al.*, 1993). Dosage for suramin starts at 20 mg/kg on a weekly basis for children whereas the adult dose is a 100–200 mg test dose then 1 g given intravenously on a weekly interval for 6 weeks.

Pentamidine, the drug of choice for early stage *Gambian* infection, is an aromatic diamidine (Sands *et al.*, 1985) that was developed from a related compound (synthalin) that induces hypoglycaemia in mammals. Although the mode of action has not been established it is observed to accumulate to high levels within the parasite, where it possibly reacts electrostatically with cellular polyanions, including the network of circular DNA that make up the mitochondrial genome (kinetoplast) (Burchmore *et al.*, 2002). While much remains unclear about the precise mechanism of action in kinetoplastids, longstanding issues like selective uptake and mechanism of (cross) resistance have now, for the most part, been resolved (Bray *et al.*, 2003). It is administered every two days by intramuscular injection for 3 weeks at a dose of 4 mg/kg for both adults and children. Pentamidine is far from ideal. The range of side effects caused by this drug includes hypotension, nausea and nephrotoxicity (Burchmore *et al.*, 2002). Diabetes mellitus is not uncommon after therapy. New classes of orally available diamidines with fewer side effects or improved central nervous system penetration are being developed (Bray *et al.*, 2003).

Melarsoprol is a dangerous drug (Stich *et al.*, 2003), one that would never achieve licensing today. This arsenical has been in use for more than half a century. It is the only

effective treatment for the chronic form of the disease caused by *Trypanosoma brucei rhodesiense*, due to its ability to cross the blood-brain barrier. A mode of action for melarsoprol is yet to be established. Cell lysis upon exposure to melarsoprol occurs rapidly. Biochemical targets of melarsoprol have been postulated to include trypanothione and glycerol-3-phosphate dehydrogenase (Burchmore *et al.*, 2002). Melarsopol has the most complicated chemotherapeutic regimen of all the sleeping sickness drugs. For adults it has traditionally been administered intravenously in three series each at 2-3.6 mg/kg per day for 3 days at intervals of 1 week., then followed by another 3.6 mg/kg per day dose for a further 3 days. This regimen must be repeated again after 2-3 weeks. For children it needs administration at 18-25 mg/kg total over a 1 month period with an initial dose of 0.36 mg/kg increasing gradually to a maximum of 3.6 mg/kg at intervals of 1-5 days. Recently, a new treatment schedule comprising of 10 daily injections of 2.2 mg/kg was shown to have a similar treatment outcome and is now in use in most areas (Burri *et al.*, 2000). The new schedule therefore offers economic and practical advantages over the standard 26-day treatment schedule, with a similar outcome. Melarsopol kills around 5-10% of patients treated due to drug enduced encephalopathy. Frequent adverse effects of this antiparasitic drug include, but are not limited to, myocardial damage; hypertension; vomiting; peripheral neuropathy; Herxheimer-type reaction, whereas the rare adverse effect is almost always shock.

Treatment failure has become an increasing problem in melarsopol therapy (Brun *et al.*, 2001; Legros *et al.*, 1999). A possible link between the emergence of melarsoprol resistance in man and imprudent use of diamidine drugs in livestock has been speculated (Barrett, 2001). Selection of resistance to veterinary trypanocides is suggested to lead to cross-resistance to drugs used in the treatment of African trypanosomiasis in man. Drug entry into the cell has been elucidated and resistance is conferred without loss of parasite viability by the loss of the unusual amino-purine transporter called P2 (encoded by the TbAT1 gene) (Carter & Fairlamb, 1993). However, deletion of TbAT1 by gene knock-out revealed only a 2-3 fold resistance to melarsen oxide *in vitro* thereby implicating other factors (Matovu *et al.*, 2003). Drug mode of action has been attributed to cell lysis due to loss of ATP which occurs as a result of glycolysis inhibition (Denise *et al.*, 2001). However, the glycolytic pathway has not yet been verified as a target for arsenical action. The debate still goes on with other targets being postulated, including glycerol-3-phosphate dehydrogenase and the low molecular weight thiol trypanothione. The link between treatment failure and resistance has yet to be established.

Because of their capacity for rapid growth within mammals, trypanosomes have been likened to some types of cancer cell (Barrett & Barrett, 2000). The inhibitor of polyamine biosynthesis difluoromethylornithine (DFMO) or eflornithine was originally developed as an anti-cancer agent (Siimes *et al.*, 1981; Jänne *et al.*, 1981). Uptake of this analogue of ornithine has been proposed to occur via passive diffusion, although a transport component has been implicated (Bacchi *et al.*, 1983; Bitonti *et al.*, 1987). It acts as a specific suicide inhibitor of the enzyme ornithine decarboxylase (ODC). Even though its specificity originates from the fact that the protozoan has an ODC that is degraded and replenished much more slowly than that of its mammalian host (Phillips *et al.*, 1987), DFMO needs to be given in large doses to be of any effect. Again, expense of treatment as well as the fact that it has no activity against *rhodesiense* sleeping sickness is a major drawback. A dosage of 400 mg/kg given by intravenous infusion in 4 equal doses (100 mg/kg every 6 hours) over the course of 2 weeks is the suggested regimen for eflornithine.

Few new drugs show promise as effective treatments against sleeping sickness (Legros *et al.*, 2002). One drug which has been developed recently and is currently in trials is the diamidine derivative DB289 (Jannin & Cattand, 2004). DB289 is only active against the early stage of the disease and will possibly be available in 2 years time (www.who.int). With the limitations of drug candidates already in the field, drug combinations are a potential method to increase the treatment options. Clinical trials of potential drug combinations have been initiated, one of which evaluated the efficacy and toxicity of three drug combinations using doses smaller than those used in monotherapy (Priotto *et al.*, 2006; Bisser *et al.*, 2007). Drug combinations have the advantage of protection from the selection of resistant strains (or at least, slow down the emergence). Combinations also allow dosage reduction of each drug, hence reducing the overall toxicity while maintaining good efficacy.

1.6 The Genome & Genomics

The gap between research and clinical need is being bridged; although not at a rate needed to provide immediate relief. Despite the medical and healthcare revolution of the late 20th century, billions of people still suffer from one or more tropical parasitic disease and the constant drain imposed by chronic sickness, loss of productive labour and premature death, imposes a multibillion dollar restriction on the economic development of the Third World (Johnston *et al.*, 1999).

The ever-emerging availability of genome sequences for hosts, vectors and numerous parasites, our rapidly expanding repertoire of computational methodologies and internet databases, functional genomics, proteomics, gene knockout/silencing protocols, and transgenesis strategies, provide the requisite tools needed to begin unravelling the intricacies of the host-pathogen-environment relationship (Christensen, 2004).

Recent technological advances have permitted the identification and validation of numerous drug targets. And the advent of molecular biology has highlighted the unique biochemical physiology in the protozoa when compared to their mammalian hosts. The Parasite Genome Initiatives are ongoing efforts of full genomic sequencing to facilitate full understanding of how parasites develop, survive and reproduce in their respective hosts, of parasite-host and parasite-immune system interactions and of the factors that determine behaviour, pathogenicity, drug resistance and antigenic variation (Johnston *et al.*, 1999).

Traditionally, the route for drug identification has been through comparative biochemistry. Biochemical pathways present in the protozoa but absent from the mammalian host should, in theory, provide targets for drug design. However, this rationale is complicated by other factors: drug entry, if the drug has to cross more than one plasma membrane, especially if one of the membrane's is mammalian in the case of *Leishmania* which reside intracellularly.

The natural progression from comparative biochemistry has been the genome sequencing projects. These provide automation and informatics, allowing rapid identification of drug targets. The parasites that cause most parasitic diseases do not pose as 'model organisms' to analyse gene function. The yeasts and nematodes like *Saccaromyces cerevisiae* and *Caenorhabditis elegans* are ideal model organisms. Most of all they are easy and cheap to maintain in the laboratory since they show rapid growth and can be obtained in very large numbers. Genome organisation is conventional and amenable to a wide variety of functional assays. Parasites often prove more difficult, needing more 'real world' maintenance (Johnston *et al.*, 1999) including animal passaging. Many cannot be cultured *in vitro*, restricting experimental manipulations. But, most crucially of all, their genomes may display complicated characteristics which make it difficult for functional analysis. Classical genetic analysis of *Trypanosoma brucei* is possible, but restricted by difficulties in obtaining large numbers of hybrid offspring from tsetse fly infections. The characteristics of individual parasitic genomes (haploid genome size, chromosome number, size, and codon content) impose limitations on the type of analysis possible (Johnston *et al.*, 1999).

Despite the limitations of the biological systems involved, the Parasite Genome Initiatives have provided a wealth of information. This, coupled with *in vivo* genetic manipulation, proves to be the most exciting prospect for parasitic diseases. Gene knockout approaches and transfection systems have become fashionable for the identification of functionally important genes. Gene knockout involves introducing a DNA construct that contains an antibiotic resistance gene, flanked by the 5' and 3' untranslated sequences from the gene to be knocked out. This leads to replacement of the gene via homologous recombination (Conway *et al.*, 2002). However, due to its complex genome repertoire, sometimes more than one round of gene knockout needs to be done. *Trypanosoma brucei* holds a unique advantage over the other kinetoplastida in that because both forms (procyclics and bloodstream forms) can be cultivated *in vitro*, selective gene replacements can be done before placing transfectants *in vivo*.

With the genome of trypanosomatid organisms now complete, a huge door has been opened that has the potential to decipher the long unanswered questions (Ash & Jasny, 2005); from RNA editing to immune evasion to antigenic variation to energy metabolism. The sequence analysis of the 11 megabase-sized chromosome of *T. brucei* revealed a 26 megabase genome containing 9068 predicted genes, including ~900 pseudogenes and ~1700 *T. brucei* specific genes (Berriman *et al.*, 2005).

Annotation of genetic information (Aslett *et al.*, 2005) is only the first step. It only allows target identification and selection of potential virulence determinants, protein antigen and drug target. Due to the complexity involved this needs to be coupled to a more in-depth analysis of metabolic pathways. The sequential interconvertions of every single compound within a cell, building up into metabolic pathways and further still into metabolic networks allows the entire picture to be seen, defining specific processes at the biological level (Fairlamb, 2002). The full cellular component of RNA molecules (transcriptome), proteins (proteome) and metabolites (metabolome) can be used to predict the full potential biosynthetic capability of an organism, in studying the response of a parasite when invading a host cell and vice-versa. The same can be applied in the discovery of potential drug targets and determining the implications associated with applied drug pressure (Breitling *et al.*, 2006).

1.7 Metabolomics

Systems biology is a rapidly emerging field. Technological advances have allowed metabolomics, the study of all metabolites within a given system, to emerge as a field

(Breitling *et al.*, 2006). Individual metabolites, and their relative abundance in different cell types, or similar cell types exposed to different environmental conditions, can be assessed by a range of techniques (Breitling *et al.*, 2006; Goodacre, 2004; Dettmer *et al.*, 2007). Arguably, the most powerful technique involves Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) (Brown *et al.*, 2005; Hirai *et al.*, 2004; Marshall *et al.*, 1998). The recently introduced Orbitrap mass analyser (Hu *et al.*, 2005) offers resolution and accuracy close to that of classical high field FT-ICR-MS (Brietling *et al.*, 2006). However, the Orbitrap operates using electrostatic rather than magnetic fields and hence by-passes the requirement of a large, super-cooled magnet. This allows it to be linked to chromatographic columns and thus avoids the risk of ion suppression effects which can occur with directly infused complex samples. This technique can resolve thousands of metabolites, in the molecular mass range of 100–1500, with extreme mass accuracy (routinely <2 ppm). This technique has been employed to build *ab initio* networks of metabolites whose masses can be linked by masses of common biochemical transformations in trypanosomes (Brietling *et al.*, 2006). Exact mass can also be used to predict likely formulae for metabolites and tentatively to identify compounds based on comparison to databases of known metabolites. The fact that multiple formulae can yield masses within 2 ppm (Kind & Fiehn, 2006) means that orthogonal approaches are required to confirm identity such as Tandem mass spectrometry (Breitling *et al.*, 2006).

In FTMS (including Orbitrap analysis), ions, generated from soft ionisation methods (e.g. electrospray) are excited into cyclotron resonances that are a function of mass and charge. The frequency of gyration of these ions is detected in the mass spectrometer and then converted to high accuracy masses using the Fourier transform. The determined mass is as a result of several different physical principles within the analyser (Hu *et al.*, 2005). The ionized metabolite mixture is trapped in an orbital trajectory of which the frequency of the orbit depends on mass/charge ratio.

In the case of the orbitrap, the trapping is achieved without the need of a magnetic field, with ions trapped in a radial electric field between a central and outer cylindrical electrode. The ultra-high resolution enables simultaneous identification of ions that would not be resolved using less accurate mass determination approaches. The combined advantage of accurate mass determination and resolution makes it possible to restrict the number of possible molecular formulae that represent an individual molecular mass (Hu *et al.*, 2005; Breitling *et al.*, 2006).

The number of metabolites present in trypanosomes is sufficiently small enough to ensure very good resolution using high resolution, high mass accuracy mass spectrometry without the need for subcellular fractionation. Metabolomics requires special approaches for sample preparation, separation, and mass spectrometric analysis (Dettmer *et al.*, 2007). The metabolome represents a vast number of components belonging to a wide variety of compound classes (amino acids, lipids, organic acids, nucleotides etc). Such diversity of compounds have reflecting diversity in their physical and chemical properties and occur in a wide range of abundances. To this effect, a careful experimental design is needed for the success of metabolomic investigations (Dettmer *et al.*, 2007). While processing samples, formation and degradation of metabolites due to remaining enzyme activity or oxidation processes is a possibility (Dettmer *et al.*, 2007). Therefore, rapid quenching and metabolism inhibition is required. Rapid quenching of metabolism with hot HEPES-buffered ethanol with subsequent freezing in liquid nitrogen has proven the most effective method of identifying metabolites in our hands (D. Wildridge, personal communication). It is necessary to also analyse culture medium without cells in order to generate spectra of medium associated metabolites to subtract from the metabolic profile. Any and every kind of preparation step will involve metabolite loss (Dettmer *et al.*, 2007). For example, the aqueous extraction employed here (chapter 5) results in poor recovery of very polar compounds as well as the loss of volatile organic compounds.

Within the metabolomics field three distinct definitions of investigations exist; metabolic profiling, metabolic fingerprinting and metabolic footprinting (Dettmer *et al.*, 2007). Profiling involves quantitative targeted metabolite analysis of metabolites of a specific class or related to a selected pathway. The disadvantage of profiling is the fact that targeted analysis is a hypothesis-driven approach rather than a hypothesis-generating one, and that the data generated is not a global view of occurrences within the metabolome. Metabolic fingerprinting involves an unbiased global screening approach. The intention is not to identify each observed metabolite but allows comparison of patterns or “fingerprints” as a result of cellular perturbations. Fingerprinting observes the true phenotype, however, the causal metabolites of the observed phenotype must be identified if any biochemical understanding is to be gained from the data. With this in mind, metabolomic fingerprinting can be used to classify or diagnose a particular perturbation. Metabolomic footprinting involves the analysis of extra-cellular metabolites in cell culture medium, which is a reflection of metabolites used or excreted by cellular processes. These three different metabolomic investigative methods shall be employed to address the question of metabolic adaptation in PCF trypanosomes (chapter 5).

1.8 Trypanosome Molecular Biology

In conjunction to the unique biochemical characteristics of trypanosomes that make attractive drug targets, other aspects of their biology including gene expression, antigenic variation, genetic diversity, are a necessity when trying to decipher the complex life cycle of these parasites.

Antigenic variation is an important aspect of African trypanosome molecular biology because this is what makes the possibility of a vaccine virtually impossible. Antigenic variation is the process by which the parasite evades the immune system of their animal host (Vickerman, 1978). Covered on the surface membrane of the parasite is a dense surface coat composed of a unique protein known as the variant surface glycoprotein (VSG) (Englund *et al.*, 1982). Recognition of this surface coat by the host immune system results in killing of the parasite. Evasion of this killing involves antigenic variation, whereby the parasite switches to the synthesis of a different variant surface glycoprotein (Barry, 1979). This indefinite process of variation is what gives rise to the relapsing parasitemia seen in patients of sleeping sickness. About 1000 VSG genes (VSGs) and pseudo-VSGs are scattered throughout the trypanosome genome (Barry *et al.*, 2005). Transcriptional activation of VSGs comes with the copying of a particular VSG into a bloodstream expression site (BES) (Horn, 2004). All expressed VSGs are located at the telomere, suggesting that VSG genes are translocated into these specific sites (Donelson, 2003). Different molecular mechanisms exist for the introduction and removal of VSG genes from these sites, but not all have been accurately characterised.

Regulation of antigenic variation is as complex as the event itself. The presence of a VSG in a sub-telomeric expression site, although necessary, does not seem to be sufficient for expression; with further events required for activation of the particular expression site (Donelson, 2003). And because only one VSG is expressed at a time, the parasite mechanism must also involve silencing of other expression sites as well as specific transcription at a particular site. Suggestions have been made for the presence of a modified base (base J) being involved in the stabilisation of repression of expression sites (Ulbert *et al.*, 2004). The genome sequence has revealed not only the location of most of the VSG genes towards the telomeric ends of chromosomes but also the fact that most VSG genes are incomplete (Barry *et al.*, 2005). Of all the identified VSGs, 95% do not properly encode protein, 5% are fully functional, 9% are atypical, 62% are full-length pseudogenes, and 19% are gene fragments. The fact that a small proportion of the total

VSG population seem to have full functional ability (as predicted from gene sequences) suggest that the possibility of incomplete genes provide a level of complexity and diversity involving mosaic gene formations.

In contrast to the bloodstream form of the African trypanosome, the procyclic form also possesses a glycoprotein coat composed of procyclins. Procyclins are first expressed in the midgut of the tsetse fly, upon bloodstream form differentiation to the procyclic form. The fact that expression of the procyclin coat is coupled to the loss of the VSG coat means a tightly regulated system is highly likely to be involved (Roditi *et al.*, 1998). Procyclin genes are organised in tandem arrays of two or three copies on two pairs of chromosomes, encoding proteins with internal dipeptide or pentapeptide repeats. Control of expression is multifactorial involving both positive and negative control elements (Matthews, 1999). Phenotype of expression is related to the ability of the trypanosome to infect the tsetse fly. Its repeat domains are protease resistant, providing a protective glycocalyx in the hydrolytic environment of the tsetse fly midgut (Gruszynski *et al.*, 2006).

Control of gene expression in trypanosomes has several novel features to it. In trypanosomes, genes contain no introns and appear packed in dense clusters along the genome, with tandem repeats of either the same or very similar genes. Gene arrays are transcribed into long polycistronic units and, uniquely, individual genes belonging to the same transcriptional unit will show significantly different expression patterns. Processing of primary transcripts into individual mature mRNAs occurs by the trans splicing of a 5' cap (a 39 nt RNA spliced leader sequence bearing a trimethyl cap) and the addition of a 3' poly(A) tail (Vanhamme *et al.*, 1995). The splice acceptor site is an AG dinucleotide immediately downstream of a polypyrimidine tract and polyadenylation sites are usually located 100-300 nucleotides upstream from the *trans* splicing signal (Clayton & Shapira, 2007).

Polyadenylation is dependent on downstream splicing which means independent processing regulation for adjacent genes are absent. This is due to the fact that poor transplicing of a particular mRNA results in poor polyadenylation of the mRNA directly upstream (Clayton & Shapira, 2007). No known polyadenylation signal exists for trypanosomes, in contrast to other many eukaryotes. The genome encodes very few potential regulatory transcription factors, meaning regulation of gene expression is post-transcriptional. With genes encoding the major surface proteins of African trypanosomes – the EP procyclin of the procyclic (insect) stage and the VSGs of the bloodstream stage being the only exception (Clayton & Shapira, 2007). It has been shown that certain

sequences in the 3'-UTR determine developmental regulation of the mRNA transcript. Indeed, regulation of an amino acid transporter in *T. brucei* has been investigated (Robles & Clayton, 2007; chapter 1.17). Investigations into regulatory sequences commonly employ cloning the intergenic region in question downstream of a reporter gene, with the reporter either as an episome or integrated into a different chromosomal location. However, this poses the problem that the regulatory sequence is removed from its usual chromosomal context, plus the fact that expression is driven by polymerases other than RNA polymerase II.

1.9 RNA Interference & Gene Knockout

The discovery of double-stranded RNA interference (dsRNAi) in *Trypanosoma brucei* provides a convenient method to generate knockout phenotypes and has allowed the advent of rapid-to-employ reverse-genetic approaches (Ngo *et al.*, 1998). The introduction of double-stranded RNA molecules in cells triggers what is thought to be a mechanism that has evolved to protect cells against undesirable RNAs (e.g. viruses or mobile genetic elements) (Clayton, 2004; Rusconi *et al.*, 2005). The mechanism is simple and, on the surface, highly specific (Figure 1-3). Introduced dsRNA proceeds through a number of sequential steps. Firstly, there is cleavage by the RNase III enzyme DICER into shorter 21-23 nucleotide dsRNA pieces termed short interfering RNAs (siRNA). These siRNAs have a characteristic phosphorylated 5' end and a two nucleotide overhang at the 3'OH end. The siRNAs then enter a RNA-induced silencing complex (RISC). A helicase, belonging to the argonaute (AGO1) family or Slicer, unwinds the two strands of the siRNA to form singlestranded RNAs (ssRNA), and RISC scans the mRNAs in the cytoplasm and cleaves the molecules that are found complementary to the RISC-contained siRNA. Hence, leading to down regulation of mRNA transcript and gene silencing.

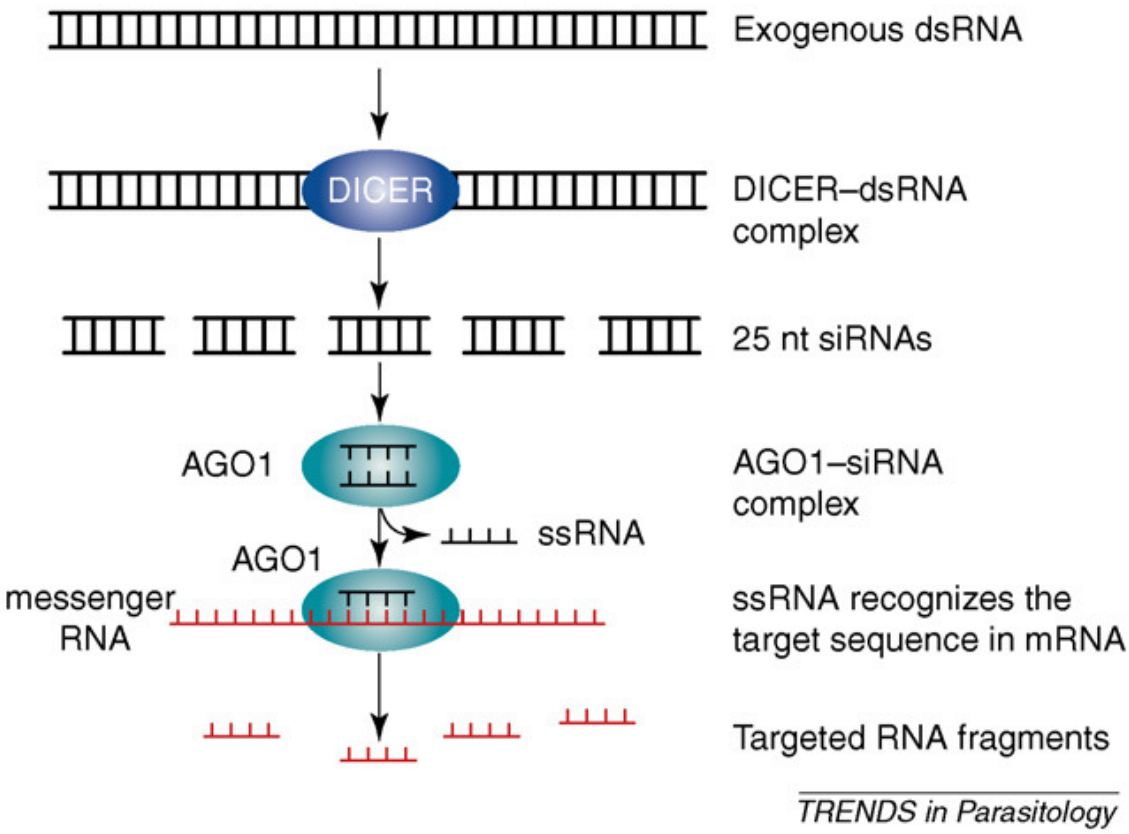


Figure 1-3: General RNAi mechanism (taken from Balaña-Fouce & Reguera, 2007).

This RNAi mechanism has been found in a number of organisms including the nematode worm *C. elegans*, flies and mammals (Fire *et al.*, 1998). RNAi potency and flexibility has allowed use of high through-put genetic screen methodologies in several organisms. Although, there is some recent debate as to the specificity of RNAi. The triggering of a number of unspecific targets is a possibility that has been postulated. The degradation by the RISC complex of partially complementary mRNA due to cross hybridisation leads to a phenotypic effect that is not due to silencing of the target gene. Reports of these phenomena have prompted scientists into assessing the specificity of RNAi silencing and posing solutions. One of which is based on the rescuing of the RNAi-mediated loss-of-function phenotype by expressing an RNAi-resistant version of the target gene (Rusconi *et al.*, 2005). Functional complementation (using an orthologue protein different in gene sequence but identical in function) offer a way to ascertain that phenotypic effects observed upon RNAi experiments are indeed due to the specific silencing of the targeted gene.

The development of tetracycline-regulated vectors for production of dsRNA has facilitated widespread use. The first RNAi experiments in *Trypanosoma brucei* used electroporation

of dsRNA into cells, leading to potent but transient phenotypes (Motyka *et al.*, 2004). The utility of RNAi was greatly enhanced with the production of vectors that stably integrate into the genome and that express dsRNA in an inducible manner. The first such vector used a tetracycline-inducible promoter to drive RNA expression. Lately, there has been development of newer vector that surround a fragment of the gene between two opposing tetracycline-inducible T7 promoters (Wang *et al.*, 2000; LaCount *et al.*, 2000). The dual T7 promoter system is applicable for both the procyclic and bloodstream forms of the parasite. It also possesses the advantage of also using a single construct to carry multiple genes, allowing simultaneous targeting (Motyka *et al.*, 2004). However, it has been noted that the two promoter system does not work for all genes (La Count *et al.*, 2000). Mutant phenotypes may not be seen due the fact that the genes chosen are non essential or because they have overlapping functions in other proteins.

The *T. brucei* Functional Genomics Project (TrypanoFAN), funded by the Wellcome Trust, aimed to utilise the information from the *T. brucei* genome project to produce a research resource and systematic collection of mutants by targeted gene inactivation using RNAi (http://trypanofan.path.cam.ac.uk/). To date, 272 gene products have been assessed by the RNAi method. This represents the first systematic analysis of gene function in a parasitic organism (Subramaniam *et al.*, 2006).

With the completion of the sequence of the megabase chromosomes of *Trypanosoma brucei*, it has become straightforward to design primers and amplify the appropriate flanking sequences to knock out genes by homologous recombination (ten Asbroek *et al.*, 1990). Homologous recombination in biology has important implications for growth, development and adaptation of all organisms. It has roles in DNA damage repair (van Gent *et al.*, 2001), for the generation of genetic diversity (Masson & West, 2001), and to ensure chromosomal segregation at meiosis (Masson & West, 2001). In African trypanosomes homologous recombination has implications in the antigenic variation processes (Conway *et al.*, 2002). Recombination is used to move new VSG genes into specialised bloodstream VSG transcription sites. Genetic and molecular evidence has suggested that antigenic variation uses homologous recombination; however the detailed reaction pathways are yet to be elucidated (Conway *et al.*, 2002). Recently the techniques of stable transformation, exploiting the natural phenomenon of homologous recombination have been successfully applied to trypanosomes (ten Asbroek *et al.*, 1990). Linearised DNA constructs, transfected into the cell, allows replacement of a gene to occur by homologous recombination. This means highly specific targeting of a chosen genomic locus. In the African trypanosome *T. brucei* a number of selectable marker genes are now employed to

achieve stable transformations (Clayton, 1999); the blasticidin (Izumi *et al.*, 1991), puromycin (Vara *et al.*, 1986), neomycin (ten Asbroek *et al.*, 1990), hygromycin B (Lee & van der Ploeg, 1991), and bleomycin (Jefferies *et al.*, 1993) antibiotic resistance genes.

The one pitfall of gene knockout experiments is to think that the product of an essential gene is automatically a good drug target. Although gene knockout removes functional protein from the cell, that level of inhibition would be hard to achieve with pharmacological agents because, rarely is 100% inhibition of target enzyme activity achieved.

1.10 Perspectives for Drug Targets

A substantial proportion of research into trypanosomes over the past two decades has been devoted to target identification and development of lead compounds that interact with these targets (Barrett *et al.*, 2003). A proven drug target is the well-known enzyme ornithine decarboxylase in *T. brucei*, inhibited by the suicide inhibitor DFMO (eflornithine) at the early stages of the polyamine biosynthesis pathway (chapter 6). Validation *in vitro* is conditional (polyamines must be of limiting availability in medium), whereas under mammalian physiological conditions (where polyamines are scarce) cell death is a certainty. Turnover for ornithine decarboxylase in *T. brucei gambiense* is less rapid than in its mammalian host (Phillips *et al.*, 1987), hence the parasite is deficient in polyamines for a prolonged period of time once the enzyme has been inactivated by DFMO. However, a functional immune system is also needed to clear parasites.

The first seven enzymes of the glycolytic pathway reside in unusual peroxisome-like organelles termed glycosomes (Michels *et al.*, 2000). Glycolysis in the bloodstream form of *Trypanosoma brucei* provides a convenient context for studying the prospects for using enzyme inhibitors as antiparasitic drugs (Bakker *et al.*, 1999; Bakker *et al.*, 1999; Eisenthal *et al.*, 1998). Glucose metabolism and the glycosome prove an attractive target, as bloodstream African trypanosomes are solely dependent on glycolysis for their ATP energy production needs. The kinetics of these glycolytic enzymes are known in detail as well as crystal structures of some enzymes of the pathway. Hence, the impetus has been “intelligent drug design” based on inhibitory molecules. However, it is usually the case that production of substrate analogues is the method of choice. This proves problematic, as novel substrate analogues usually do not out-compete the natural substrate. Binding is never much tighter than natural substrates and inhibition is ineffective.

Hexose analogues may represent good compounds to inhibit glucose metabolism (chapter 4) in trypanosomes. Several sugar analogues were developed in order to test the limits of the structure-activity relationship of the THT1 hexose transporter in bloodstream trypanosomes (Azema *et al.*, 2004). THT1 is the principal hexose transporter exhibited by bloodstream form *Trypanosoma brucei*. THT1 itself proves an attractive cellular target because it differs markedly from all of the mammalian GLUT transporters (Tetaud *et al.*, 1997).

There are two basic metabolic methods of killing an organism; either flux through an essential metabolic pathway can be decreased to the point where life is no longer possible, or a metabolite's concentration can be increased to toxic levels (Eisenthal *et al.*, 1998). The difference being that decreasing flux need not be accompanied by large changes in metabolite concentration and increases in metabolite concentration may not necessarily lead to changes in flux. It all depends on the integrated enzyme system involved.

Glucose is also metabolised by a second route, the pentose phosphate pathway (PPP); a pathway that does play a crucial role both in the metabolism of many parasitic protozoa and in the host's relationship with these organisms (Barrett, 1999). The pentose phosphate pathway of trypanosomes poses another attractive target mainly because of the fact that enzymes of that pathway are more closely related to cyanobacterial isoforms than they are to eukaryotes (Barrett *et al.*, 2003). The PPP serves to convert glucose 6-phosphate (G6P) to ribose 5-phosphate (R5P), which is used in nucleotide biosynthesis. In some systems, it also provides other important phosphorylated carbohydrates, such as erythrose 4-phosphate (E4P), which serves as a precursor in the synthesis of aromatic amino acids and vitamins, as well as sedoheptulose 7-phosphate (S7P), an important component of some bacterial cell walls. The other key product of the pathway is NADPH, which serves as a hydrogen donor in reductive biosynthesis, and plays an integral role in the defence against oxidative stress.

The enzyme 6-phosphogluconate dehydrogenase may prove to be a potential target for chemotherapy. This has been elucidated from the fact that in other eukaryotic organisms, absence of the 6PGDH gene has been shown to be conditionally lethal (Barrett, unpublished). The *Trypanosoma brucei* 6PGDH gene has been cloned and its enzyme purified (Barrett, 1994). Subsequently, it has been found to not only have a number of key kinetic features that distinguish it from its mammalian counterpart but also interact with suramin and trivalent aromatic arsenoxides (Hanau *et al.*, 1996). Recently, three series of inhibitors have been designed for this target. These include phosphorylated carbohydrate substrate and transition state analogues, non-carbohydrate substrate analogues and also

triphenylmethane-based compounds. All have shown selective inhibition of the trypanosomal 6-phosphogluconate dehydrogenase and representatives of each have trypanocidal activity (Hanau *et al.*, 2004). More recently, crystal structures of recombinant *Lactococcus lactis* 6-phosphogluconate dehydrogenase (LlPDH), which shares significant sequence identity with the enzymes from sheep liver and the protozoan parasite, have been determined (Sundaramoorthy *et al.*, 2007). Studies through complex with substrate, cofactor, product and inhibitors showed high conservations of key residues in the active site, allowing LlPDH to serve as a model system for further structure-based inhibitor design.

Thiol metabolism is also a much studied system in trypanosomes (Comini *et al.*, 2004). The role of glutathione in most eukaryotes, is undertaken by a unique low molecular weight thiol called trypanothione (N^1,N^8-bis-glutathionylspermidine). Low molecular weight thiols contribute to defence against reactive oxygen species, which arise internally through the normal respiration or from the host. The biosynthetic pathway of trypanothione consists of two steps; formation of glutathionylspermidine from spermidine and glutathione by glutathionylspermidine synthetase, and the formation of trypanothione from glutathionylspermidine and glutathione by trypanothione synthetase (Fairlamb *et al.*, 1990). The enzymes of trypanothione biosynthesis are also potential targets for drug design (Schmidt *et al.*, 2002), due to the fact that dithiol trypanothione has many downstream cellular processes including DNA synthesis, ascorbate regulation, and detoxification of hydrogen peroxides.

1.11 Drug Uptake via Nutrient Transporters

Drug uptake via nutrient transporters is the one field that has received a lot of interest in recent times (Barrett & Gilbert, 2006). *Trypanosoma brucei*, in comparison to the other trypanosomes which reside intracellularly, is in the privileged position of being exposed in the bloodstream. Privileged from a drug design point of view meaning potential drugs do not have to cross mammalian cell membranes prior to reaching their target. A dense coat of variant surface glycoprotein (VSG) prevents complement from reaching the membrane and immunoglobulin from “seeing” membrane proteins (Borst & Fairlamb, 1998). There is sufficient space between VSG dimers to allow low molecular weight compounds to reach transporters in the membrane. This has the added advantage of allowing selective targeting via routes of entry that are unique to trypanosomes. This concept has been highlighted in

the case of the TbAT1 nucleoside transporter, P2 (Barrett & Gilbert, 2006; Carter *et al.*, 1995; Carter & Fairlamb, 1993).

The role of the P2 transporter was discovered when it was found that *Trypanosoma brucei* cultures selected for resistance to melamine based arsenicals had lost this transporter (Carter & Fairlamb, 1993). The trypanosomal gene *TbAT1* has been found to encode the P2 adenosine/adenine transporter (Maser *et al.*, 1999; Matovu *et al.*, 2003). Diamidine and melamino phenyl arsenical entry into the cell, via this transporter, is due to the similarity in recognition motifs between diamidines and arsenicals and the transporter's normal amino-purine substrates (Tye *et al.*, 1998; Barrett & Fairlamb, 1999; de Koning *et al.*, 1999). The 6-amino group of the purine ring, attached to a carbon, in turn bound via a double bond to a nitrogen is crucial for recognition. Identification of this recognition motif has led to the development of cytotoxic molecules with that motif grafted on (Tye *et al.,* 1998). Several compounds have been identified through the empirical route and are getting some use (Barrett *et al.*, 2002). For example, the triazine derivative SIPI 1029 is used in China against *Trypanosoma evansi* infections in domestic livestock, as well as another inhibitor of polyamine biosynthesis: CGP40215, which interacts via S-adenosylmethionine decarboxylase.

In general, exploitation of nutrient transport systems for delivery of new drugs needs to confer to several key conditions; namely selectivity and efficacy. More specifically, there needs to be high affinity of the trypanocide for the particular transporter, coupled with low affinity for the mammalian transporter. Furthermore, there needs to be low abundance of competing substrates and ideally there needs to be concentrative rather than equilibrative uptake (Hasne & Barrett, 2000; Barrett & Fairlamb, 1999; De Koning *et al.*, 1999). The P2 purine transporter, thus far, appears to satisfy these two conditions but has one major disadvantage: the fact that the parasite can lose this transporter without compromising viability. Therefore, developing resistance without a loss in virulence. Hence, new routes of drug delivery are essential.

1.12 Transport Phenomena across Membranes

A cell or an organelle cannot be either wholly open or wholly closed to its surroundings. Its interior must be protected from certain toxic compounds, and yet metabolites must be taken in and waste products removed. As well as uptake of nutrients, all communication among cells and between cells and their environment must occur through the membrane interface. Biological membrane lipid bilayers allow selective permeability. The double

hydrocarbon tail of the lipid molecules allows bilayer formation rather than the micelle formation that would occur with the single tail. Lipids are the major constituents of biological membranes with glycerophospholipids, sphingolipids, glycosphingolipids, and glycoglycerolipids the four main classes of membrane-forming lipids. Membrane fluidity is essential for all living cells. At low temperatures, the head groups and hydrocarbon tails pack closely, forming an almost solid gel state. As the temperature increases the membrane "melts" and becomes thinner as a more fluid state is adopted.

For essential small molecules, such as amino acids, sugars and ions, crossing the plasma membrane is through the action of integral membrane protein pumps, channels, or transporters. Internalisation of particles, macromolecules and solutes occurs by endocytosis. This involves the membrane folding around material on the outside the cell (Conner & Schmid, 2003). Vesicles are then pinched off from the cell surface so that it lies within the cell. Endocytosis can be separated into pinocytosis and phagocytosis. These two can be distinguished from one another by the size of particle ingested and by its dependence on actin polymerization (Liu & Shapiro, 2003). Phagocytosis can be described as "cell eating" (the uptake of large particles) whereas pinocytosis or "cell drinking" can be described as the uptake of fluids and solutes.

Three categories of transport across biological membranes exist – passive, facilitated and active. All have quite different properties and serve different purposes for the cell. There is an equilibrium approached by transport across any membrane. That is, a substance that can traverse the membrane will eventually reach the same concentration on both sides, meaning no net transport. This equalisation can only be circumvented under certain conditions; preferential binding and subsequent modification of substrate upon transport, membrane potential that influences the distribution of ions, or coupling of a thermodynamically favoured process to transport.

It has been said that transporters cannot catalyse an increase in the rate of biomembrane transport to near that which would occur if simple diffusion across the membrane was possible (Van Winkle, 1999). Passive transport is essentially diffusion and occurs via the random motion of molecules (Brownian motion). This leads to equilibrium of free concentration across the membrane. The net rate of transport depends on the concentration difference across the membrane – the higher the difference, the higher the net rate. However, with hydrophilic substances, diffusion occurs very slowly due to the insolubility of hydrophilic substances within the membrane itself.

Facilitated transport, sometimes called accelerated diffusion, occurs via two methods – pores, formed by transmembrane proteins, or by carrier molecules. Pore formation increases the diffusion rate by a large order of magnitude. Carrier facilitated transport effectively increases the solubility of its substrate in the membrane. Flow is never directed and thus can occur in both directions. The distinguishing feature of both types of accelerated diffusion methods is the fact that carrier facilitated diffusion is a saturable process, due to the fact that there is a limited number of carrier molecules within a membrane system. Whereas the rate of passive diffusion can increase linearly with the concentration difference.

Active Transport is transport of a substrate against its concentration gradient and this requires a free energy source. The free energy source comes from the hydrolysis of ATP. Coupling of the energy to the transport process can either be direct or indirect. Ion pumps directly couple the hydrolysis of ATP to transport, with a subunit of the protein being enzymatically active. The pump exists in two conformational states – open to the cytosol and open to the cell exterior. Transition between the states is directly linked to the free energy change. Transition to the cytosol-open state is triggered by the binding of ATP and release of phosphate, whereas transition to the outside-open state occurs when ADP is released.

The other form of active transport does not depend directly on ATP but employs the hydrolysis of ATP in an indirect way. In this instance, a favourable gradient is used to drive the transport of another molecule against its unfavourable concentration gradient. Transport of both molecules can occur in the same direction (symport) or in opposite directions (antiport).

One other method that achieves transport against a concentration gradient is transport by modification. Upon moving into a cell by diffusion the molecule is modified in a way that prohibits its exit through the membrane. The modified molecule accumulates within the cell but the apparent concentration gradient is maintained.

1.13 Amino Acid Transporters

Protein phylogeny, based upon primary amino acid sequence relatedness, reflects the evolutionary process and therefore provides a guide to structure, mechanism and underlying function (Chang *et al.*, 2004). Any two proteins that are related by a common descent are expected to exhibit similar structures and functions to a degree proportional to

sequence similarity. The transporter classification system (TC) (http://www.tcdb.org) has been adopted based on similarity between transporters (Busch & Saier, 2003; Busch & Saier, 2004). This employs computational methods to determine distant relationships. In general, transporter types can be distinguished according to phylogeny, substrate specificity, transport mechanism and cell specificity. Amino acids and their derivatives are known to be transported by 21 families of secondary carriers, 13 families of ATP-dependent transporters, and 3 families of channel proteins (Saier, 2000).

The primary modes of transport with transporters of amino acids and their derivatives have always been thought to involve channels and carriers (Saier, 2000) with most being secondary carriers. Mechanisms of channel transport are well understood whereas the carrier system is still unclear. This, in large part, is due to the availability of high-resolution three-dimensional structural data for channel systems.

Work by Saier has provided a comprehensive guide to the diversity of carriers that mediate the transport of amino acids across membranes. The range of substrates transported by an individual member of a family can be narrow or broad and structural similarity in substrate is not a requirement. With regards to energy-coupling mechanisms employed for transport, amino acid transporters use chemical energy in the form of ATP and electrochemical energy stored in favourable ion gradients (H^+, Na^+). Syntheses, activities, and degradation of amino acid transport proteins are similar to the mechanisms employed for other transport proteins and cytoplasmic enzymes. Classification and characterisation of transporter families from studies have shown underlying evolutionary traits. Transport mode and energy coupling mechanisms are highly conserved, protein topology, polarity of transport and substrate specificity are conserved to an intermediate degree, whereas the regulatory mechanisms imposed on transporters are poorly conserved (Saier, 2000).

Conserved amino acid transporters among three sequenced eukaryotic genomes – *Saccharomyces cerevisiae*, *Arabidopsis thaliana* and *Homo sapiens* showed transporters belonging to five different superfamilies: amino acid/polyamine/organocation transporter superfamily (APC), sodium/dicarboxylate symporter superfamily (SDS), neurotransmitter superfamily (NTS), amino acid transporter superfamily 1 (ATF1), and the amino acid transporters within major facilitator superfamily (MFS) (Wipf *et al.*, 2002).

Classified among the transporter superfamilies is the amino acid/polyamine/organocation (APC) superfamily, which includes proteins that function as solute-cation symporters and solute-solute antiporters. APC family members are not highly specific but instead transport

several related, or even a wide spectrum of structurally different amino acids including sometimes D-isomers (Wipf *et al.*, 2002). APC transporters are ubiquitous; homologues are found in animals, plants and bacteria but are best understood in yeast. Amino acid transport mediated by members of the APC family is diverse; has functions in uptake and nutrition and is ion coupled with all members catalysing transport using a monovalent cation symport mechanism (Chang *et al.*, 2004). In multicellular organisms they might function as exchangers for both selective accumulation of specific amino acids, and redistribution and homeostasis of the intracellular concentrations. The majority of proteins exhibit uniform topology with twelve transmembrane α-helical domains (Jack *et al.*, 2000) although exceptions to this trend do exist.

Distantly related to the APC superfamily are the amino acid/auxin permease family (AAAP) and the hydroxyl and aromatic amino acid porter family (HAAAP). The AAAP family includes hundreds of proteins from plants, animals, yeast and fungi. They exhibit very broad specificities as well as transporting D-isomers. The proteins have 11 putative transmembrane spanning domains and show limited sequence similarity with members of the large APC superfamily (Saier, 2000). The HAAAP family is found exclusively in bacteria, transporting aromatic amino acids, but show topological features common to members of the eukaryotic AAAP family.

The sodium/dicarboxylate symporter superfamily (SDS) and neurotransmitter superfamily (NTS) are exclusive to animal genomes, with no homologues found in yeast or plant genomes. SDS members mediate electrogenic glutamate and aspartate uptake and their proteins contain 10 putative membrane spanning domains (Wipf *et al.*, 2002). NTS members share a common structure with 12 transmembrane domains and include membrane carriers for γ-aminobutyric acid (GABA), proline and glycine.

ATF1 family members were first seen in plants with homologues later found in yeast and animals. Membrane domains differ from 9-11 segments. This superfamily contains plant-specific sub-branches and branches that are more related to yeast and human transporters (Wipf *et al.*, 2002).

Within two subfamilies of the major facilitator superfamily (MFS) amino acid transporters were found. MFS proteins transport a wide spectrum of substances unrelated to amino acids, including sugars, polyol drugs, neurotransmitters, phosphorylated glycolytic intermediates, peptides, nucleosides and organic anions (Saier, 2000). They employ multiple transport mechanisms such as uniport or hydrogen ion cotransport (Wipf *et al.*,

2002). They are ubiquitous in all three domains of organism and each of the 29 currently recognised families is specific for one class of compound.

1.14 Human Amino Acid Transporters

Mammalian amino acid transporters consist of a complex system allowing amino acids to be transported through a number of different carrier-proteins exhibiting overlapping specificity. In general, they are divided into Na^+-dependent and Na^+-independent groups. The mammalian amino acid carrier proteins belong to different gene families, exhibit overlapping but distinctive substrate specificities, and can further be distinguished by their requirement for the co-transport or counter-transport of inorganic ions (Closs *et al.*, 2004). The main functional criteria used to define amino acid transporters in mammals are substrate specificity and the thermodynamic properties of transport (Palacin *et al.*, 1998).

One of the first amino acid transport systems described for mammalian cells was a Na^+-independent system termed **System L** (Kilberg *et al.*, 1993), named 'L' because of its high affinity for leucine, it is also able to transport large apolar branched amino acids, catalysing exchange of amino acids rather than net uptake.

System A, originally described four decades ago is a pH-dependent transporter of alanine, glycine and proline (Oxender & Christensen, 1963). The activity of this system is pH dependent and is expressed in response to substrate deprivation. There is the suggestion that it provides the concentrations of cytosolic amino acids necessary to drive the uptake of other amino acids by exchange through other systems. Unusually, System A recognises N-methylated amino acids and can tolerate the substitution of Na^+ with Li^{2+} (Christensen *et al.*, 1965). Other homeostatic roles have been implicated in its repertoire and it has been shown that in many cell types its activity is highly regulated, including upregulation during cell-cycle progression and hormonal control (Palacin *et al.*, 1998).

System Gly, first identified in isolated rat hepatocytes that were shown to contain a glycine specific system (Christensen & Handlogten, 1981), occurs in several cell types (Eavenson & Christensen, 1967). Glycine and sarcosine are the two known substrates for this system with a strict dependence on Na^+ and Cl^- availability. The hepatic System Gly is Na^+-dependent and appears to transport two sodium ions for each glycine (Kilberg *et al.*, 1993).

System N, called System N to reflect an apparent affinity for neutral amino acids containing nitrogen-bearing side chains, is involved in the Na^+-dependent uptake of glutamine and histidine and other amino acids with nitrogen in their side chain (Kilberg *et al.*, 1980). Present mainly in liver cells, it has been suggested that a System N-like activity is an important component of histidine-induced gastric acid secretion. It has high pH sensitivity and resembles **System A** in sequence but differs in mechanism (Reimer *et al.*, 2000).

System ASC shows variable pH dependency for transport of the amino acids alanine, serine, cysteine, and threonine but also recognises other aliphatic amino acids. Although Na^+ dependent, System ASC appears to mediate amino acid exchange rather than net uptake (Reimer *et al.*, 2000).

System β is used to transport taurine and alanine in erythrocytes, cardiac muscle and kidney, with differences in substrate affinity and specificity (Miyamoto *et al.*, 1990).

Systems y^+, **$B^{0,+}$**, **$b^{0,+}$**, **b^+** and **y^+L** are the five transport systems that mediate the uptake of cationic amino acids (Palacin *et al.*, 1998). **System y^+** is the most widespread, transporting lysine, histidine and arginine in a selective sodium independent manner. The rest have been described only in specific tissues. **System $B^{0,+}$** is found in only blastocysts and oocyte fibroblasts and transports alanine, valine and lysine. **System $b^{0,+}$** shows similarity to **System $B^{0,+}$** but is only found in kidney, small intestine and blastocysts. **System y^+L** transport neutral amino acids, with Na^+ dependency, while for lysine transport there is no sodium requirement. **Systems $b^{0,+}$** and **y^+L** are the result of activity of heteromeric amino acid transporters, meaning transporter activity is elicited by coexpression of a heavy and light subunit (Palacin *et al.*, 2001).

Conversely, the two acidic amino acids, glutamate and aspartate are transported into mammalian cells via five different transporters: **ASC**, **GLT-1** (Glutamate transporter 1), **GLAST** (Glutamate/aspartate transporter), **EAAT4** (excitatory amino acid transporter 4) and **EAAC1** (excitatory amino acid carrier 1). Recently, it has been found that glutamate transporters in the mammalian central nervous system exhibit glutamate-gated chloride-channel activity in addition to glutamate-transport activity (Slotboom *et al.*, 2001). The physiological function of this chloride-channel activity is still unknown, however, an excitatory response might be a consequential result. The transporters remove the excitatory neurotransmitter glutamate from the synaptic cleft driven by the sodium/potassium concentration gradient.

Mammalian cells express many amino acid transport systems which differ in mechanism and substrate specificity (Ganapathy *et al.*, 2004). Many are needed because no single transport system can satisfy the structural requirements to take up all the different amino acids. The fact that amino acid requirements differ for each cell type means that expression profiles of transport systems will also differ. The potential for any given amino acid transport system will depend on its substrate specificity and its tissue expression profile (Ganapathy & Ganapathy, 2005). Different cells contain a distinct set of transport systems, as a combination of common or almost ubiquitous (Systems A, ASC, L, and y^+) and tissue-specific (systems $B^{0,+}$, N^m, and $b^{0,+}$) transport systems (Palacin *et al.*, 1998). The vast range of amino acid transporters with overlapping substrate specificities means uptake of a particular amino acid within a given type of cell is always as a function of many different transporters.

1.15 Amino Acids & Trypanosomes

In yeast, animals and plants amino acids play an essential role in various processes, including protein synthesis, hormone metabolism, cell signalling, nerve transmission, cell growth, osmoregulation, cell cycling, production of metabolic energy, nucleobase synthesis, nitrogen metabolism and urea biosynthesis. The digenetic life cycle of *T. brucei*, which involves a complex development of morphologically distinct forms in the insect vector and the mammalian host, indicates that the parasites are exposed to ever changing environments (Besteiro *et al.*, 2005). Of the three trypanosomatids, *T. brucei* has the most restrictive metabolic repertoire, reflecting its extracellular bloodstream form life cycle (Berriman *et al.*, 2005), which has greater access to nutrients in the plasma. Trypanosomes lack biosynthetic pathways for the essential amino acids and the procyclic form requires exogenous proline as an energy source.

Plants and fungi can synthesise each of the 20 amino acids by using biosynthetic pathways inherited from their bacterial ancestors and the ability to synthesise the nine amino acids (Phe, Trp, Ile, Leu, Val, Lys, His, Thr, and Met) was lost in a wide variety of eukaryotes that evolved to feed on other organisms (Payne & Loomis, 2005). The genome of the African trypanosome (Berriman *et al.*, 2005) has revealed that most of the enzymes of the classical pathways for aromatic amino acid oxidation are missing. Conversion of phenylalanine to tyrosine does not occur in *T. brucei*, due to the fact that it does not have the enzyme phenylalanine-4-hydroxylase. All trypanosome species have genes for transamination and reduction to the corresponding aromatic lactate derivative. Studies have

found that aromatic amino acids were the preferred amino donors for the transamination of α-ketomethiobutyrate to methionine (Berger *et al.*, 1996). The transamination of aromatic amino acids is essential to regenerate methionine from α-ketomethiobuytrate (KMTB), an end product of S-adenosylmethionine from the synthesis of polyamines.

Catabolism of histidine is absent in *T. brucei* and a functional urea cycle is missing across the trypanosomes. Branched chain amino acids can be converted to acetyl-CoA derivatives within the mitochondria.

In *T. brucei*, threonine is not oxidised via the 2-oxobutyrate pathway, instead being degraded to acetyl-CoA and glycine by a mitochondrial specific threonine dehydrogenase. Threonine has been found to be a precursor for acetate (which feeds into lipid biosynthesis, Figure 4) in *Trypanosoma brucei* (Cross *et al.*, 1975). There is cleavage of threonine to form glycine and acetate within the mitochondrial compartment. Preliminary examination of changes in medium amino acid levels during growth of *Trypanosoma brucei* revealed complete utilisation of threonine (Cross *et al.*, 1975).

Alanine was found to be the most abundant amino acid present in *T. gambiense* (Chappell *et al.*, 1972). Cysteine is an essential growth factor for bloodstream form trypanosomes and is involved in protein biosynthesis as well as in the production of glutathione and trypanothione (Duszenko *et al.*, 1992). Cysteine essentiality was discovered by mishap when cysteine was added into the culture medium instead of cystine (Cross & Manning, 1973). In a mammalian culture system, feeder cells can take up cystine and excrete cysteine, which is otherwise toxic to the cells being cultured. Cross & Manning demonstrated that the addition of cysteine eliminated the feeder-cell requirement for *T. brucei* culture.

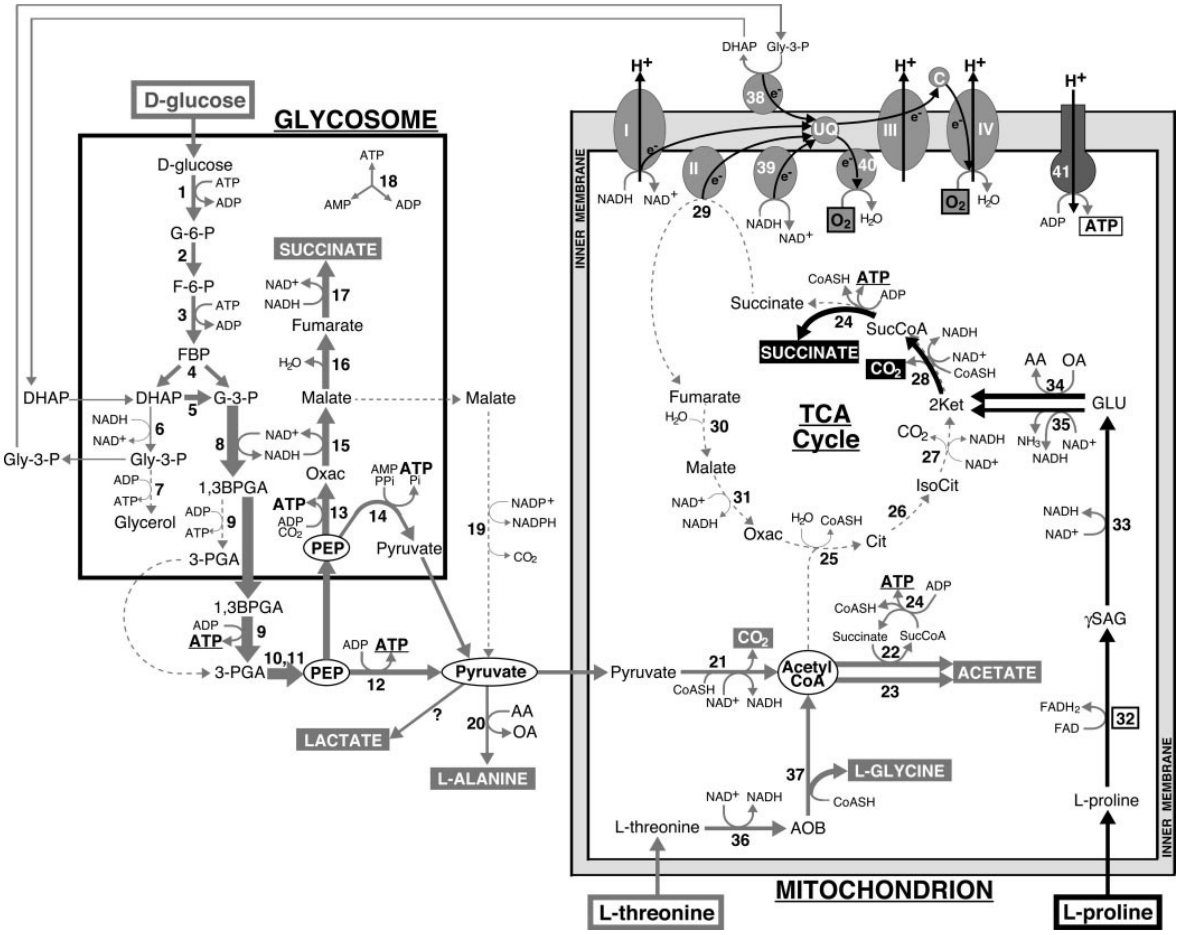


Figure 1-4: Schematic representation of carbon source metabolism in the procyclic form of *T. brucei* (taken from Lamour *et al.*, 2005).

Enzymatic steps of D-glucose and L-threonine metabolism are represented by gray arrows, while those for L-proline are represented by black arrows. Excreted end products (acetate, L-alanine, L-glycine, lactate, succinate, and CO_2) are in white characters on a gray background (from D-glucose and L-threonine metabolism) or on a black background (from L-proline metabolism). The metabolic flux at each enzymatic step is tentatively represented by arrows with different thicknesses. Dashed arrows indicate steps which are supposed to occur at a background level or not at all, under the standard growth conditions (glucose-rich medium). The enzymatic reaction leading to the production of lactate (possibly from pyruvate) is not known and is indicated by a question mark. The glycosomal and mitochondrial compartments and the tricarboxylic acid cycle (TCA) are indicated. The underlined and boxed ATP molecules are produced by substrate level phosphorylation and oxidative phosphorylation, respectively. The circled metabolites (phosphoenol- pyruvate (PEP), pyruvate, and acetyl-CoA) are located at a branching point. Abbreviations: AA, amino acid; AOB, amino oxobutyrate; 1,3BPGA, 1,3-bisphosphoglycerate; C, cytochrome c; Cit, citrate; CoASH, coenzyme A; DHAP, dihydroxyacetone phosphate; F-6-P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; G-3-P, glyceraldehyde 3-phosphate; G-6-P, glucose 6-phosphate; GLU, glutamate; Gly-3-P, glycerol 3-phosphate; IsoCit, isocitrate; 2Ket, 2-ketoglutarate; OA, 2-oxoacid; Oxac, oxaloacetate; 3-PGA, 3-phosphoglycerate; Pi, inorganic phosphate; PPi, inorganic pyrophosphate; γ-SAG, glutamate γ-semialdehyde; SucCoA, succinyl-CoA; UQ, ubiquinone pool. Enzymes are as follows: 1, hexokinase: 2, glucose-6-phosphate isomerase; 3, phosphofructokinase; 4, aldolase; 5, triose-phosphate isomerase; 6, glycerol-3-phosphate dehydrogenase; 7, glycerol kinase; 8, glyceraldehyde-3-phosphate dehydrogenase; 9, phosphoglycerate kinase; 10, phosphoglycerate mutase; 11, enolase; 12, pyruvate kinase; 13, phosphoenolpyruvate carboxykinase; 14, pyruvate phosphate dikinase; 15, glycosomal malate dehydrogenase; 16, glycosomal fumarase; 17, NADH-

dependent fumarate reductase; 18, glycosomal adenylate kinase; 19, malic enzyme; 20, alanine aminotransferase; 21, pyruvate dehydrogenase complex; 22, acetate:succinate CoA-transferase; 23, unknown enzyme, possibly acetyl-CoA synthetase; 24, succinyl-CoA synthetase; 25, citrate synthase; 26, aconitase; 27, isocitrate dehydrogenase; 28, 2-ketoglutarate dehydrogenase complex; 29, succinate dehydrogenase (complex II of the respiratory chain); 30, mitochondrial fumarase; 31, mitochondrial malate dehydrogenase; 32, proline dehydrogenase; 33, pyrroline-5 carboxylate dehydrogenase; 34, glutamate aminotransferase; 35, glutamate dehydrogenase; 36, L-threonine dehydrogenase; 37, acetyl-CoA:glycine C-acetyltransferase; 38, glycerol-3-phosphate oxidase; 39, rotenone-insensitive NADH dehydrogenase; 40, alternative oxidase; 41, F_0/F_1-ATP synthase; I-IV, complexes of the respiratory chain.

Proline is the principal source of carbon and energy of procyclic forms provided glucose is absent (Lamour *et al.*, 2005; Evans & Brown, 1972; L'Hostis *et al.*, 1993). The proline ring is opened up and oxidised to α-ketoglutarate and subsequently to succinate which forms part of the Kreb's Cycle (Figure 1-4). Proline has also been implicated in the differentiation from the non-infective to the infective stage within the insect vector (Contreras *et al.*, 1985). Within the tsetse fly, proline is the principal energy source (Balogun, 1974), as the digestive contents of the gut are rich in amino acids but lacking in glucose. Proline metabolism has been studied in procyclic form *Trypanosoma brucei* and its implications with glucose availability are intertwined (Lamour *et al.*, 2005; chapter 4). Trypanosomes seem to be able to use proline as an energy source in the absence of glucose. Similarly, *Leishmania donovani* has adapted to the extreme changes in proline environments, from the sandfly gut to the mammalian intracellular environment. Three proline transport systems have been identified in axenic cultures of *Leishmania donovani* (Marazeb *et al.*, 1999). Systems A and B are cation dependent and independent in promastigotes, respectively. Whereas, System C is cation independent in amastigotes. Proline uptake in procyclic forms of the African trypanosome has been shown to be carrier mediated with an apparent K_m of 21 +/-2.9 μM and a V_{max} of 7.0 nanomoles/min/10^8 cells. This level of uptake is modulated by the presence or absence of glucose in the growth media (Lamour *et al.*, 2005, chapter 4).

Methionine is involved in protein synthesis and is a primary sub-unit source in the polyamine biosynthetic pathway. Methionine uptake in procyclic and bloodstream form trypanosomes has been shown to be mediated by a similar transporter with a high affinity (Hasne & Barrett, 2000). Exogenous methionine is adenosylated by S-adenosylmethionine synthase to form S-adenosylmethionine (AdoMet). AdoMet is a key metabolite in transmethylation reactions and its decarboxylated derivative provides aminopropyl group in polyamine synthesis. AdoMet also plays a role as the alternative source of adenosine.

The by-product of polyamine synthesis is 5'-methylthioadenosine (MTA). MTA is enzymatically degraded to adenine and 5'-methylthioribose-1-phosphate, a carbohydrate intermediate that is recycled to methionine in the methionine cycle (Goldberg *et al.*, 1997; Berger *et al.*, 1996).

African trypanosomes have been proposed to exhibit a dedicated transporter for S-adenosylmethionine that is independent of methionine uptake (Goldberg *et al.*, 1999). This has been shown by challenging uptake of AdoMet with methionine and compounds which compete with adenosine for transport. Goldberg *et al* found that neither methionine nor adenosine analogues significantly inhibited AdoMet uptake. However, certain aspects of their methodology do not stand up to criticism. Challenging compounds were never in excess within the uptake assay (Goldberg *et al.*, 1997). A unique transporter, highly specific for AdoMet, was not confidently shown and was only assumed from other compounds (not in excess concentrations) not inhibiting AdoMet uptake.

1.16 Amino Acid Transporters in Trypanosomes

The metabolism of the protozoan parasites of the *Trypanosoma* and *Leishmania* genus is, in general, strongly based on amino acid consumption (the BSF African trypanosome being an exception). Amino acid transporters are key to this process. However, amino acid transporters can provide another function – as environmental sensors (Holsbeeks *et al.*, 2004). The duality of the life cycle of these parasites requires that changes in its surrounding environment be observed. Membrane proteins, including transporters, are the first cell proteins to come into contact with its surrounding environment and therefore provide the cues for many downstream cell processes.

Most work in the area of amino acid transporters in trypanosomatids only deals with general uptake and biochemical characterisation of uptake. Only recently have researchers gone on to isolate or characterise transporters or transport systems. RNAi and gene-knockout are methods that allow a specific gene to phenotype characterisation, yet have gone unused by much of the research into amino acid transport to date.

Since around 5-10% of the genes of most organisms encode membrane transporters, there is likely to be in the order of 400 membrane transporters in *Trypanosoma brucei*. Amino acid transporters constitute one of the largest families of permeases in the genome (Berriman *et al.*, 2005). A family of amino acid transporters exist with overlapping substrate specificities, encoded by 46 genes, as found from the *Trypanosoma brucei*

genome project. One amino acid transporter, *TbAATP1*, has been preliminarily characterised by RNAi (Accoceberry & Barrett, unpublished data). This transporter has been shown to be a transporter of small uncharged amino acids (serine, threonine, alanine, glycine, cysteine and asparagine), with uptake of these amino acids being repressed in cell lines that contained down-regulation of this transporter (chapter 3). With the use of uptake assays to screen potential trypanocidal amino acid analogues and RNAi cell lines of parasites with reduced transporter activity, more insight into the role of this transporter can be gained, not only in the identification of trypanocidal properties of amino acid analogues but also in the uptake mechanisms involved.

Work has also been published on the family of amino acid transporters in *Leishmania* and *Trypanosoma cruzi*. Work done on *Leishmania donovani* revealed genus specific motifs as well as motifs common across the trypanosomatidae family (Akerman *et al.*, 2004). A large number of amino acid transporters have been located in the *T. cruzi* genome (Bouvier *et al.*, 2004). The tandem organisation of such genes seems to be a feature common to trypanosomatidae. Whereas in *Trypanosoma brucei* there is indication of the presence of a family of amino acid transporters with broad substrate specificity, the substrate specificities for *T. cruzi* and *Leishmania* remain unclear, although in the case of *Leishmania*, developmental regulation has been shown to be exhibited for one amino acid transporter gene (Geraldo *et al.*, 2005) as well as for general proline transport (Mazareb *et al.*, 1999).

In *Trypanosoma brucei*, amino acid transport has been studied since the 1970's. These studies highlighted the potential of the amino acid transport system as drug targets. The aim was to separate various trypanosomal carriers from each other by kinetic means, showing a transport system specific for neutral amino acids (Owen & Voorheis, 1975). Amino acid transport was divided into certain systems; the neutral amino acid carrier mediating transport of glycine, alanine, serine and threonine, the second neutral amino acid carrier mediating transport of valine, leucine, proline and methionine, the first acid amino acid carrier mediating transport of glutamic acid, and the first basic amino acid carrier mediating lysine transport (Voorheis, 1973). With the vast information now available on the genome we know there is a more complex story at play, with many transporter genes showing probable variable expression and broad substrate specificity.

1.17 Genes of the Amino Acid Transporter Family

The amino acid transporter family constitutes one of the largest families of permeases in *T. brucei*. From the genome database 46 amino acid transporter genes were identified (including 3 pseudogenes) in *T. brucei*, however, only 38 were initially annotated (Berriman *et al.*, 2005).

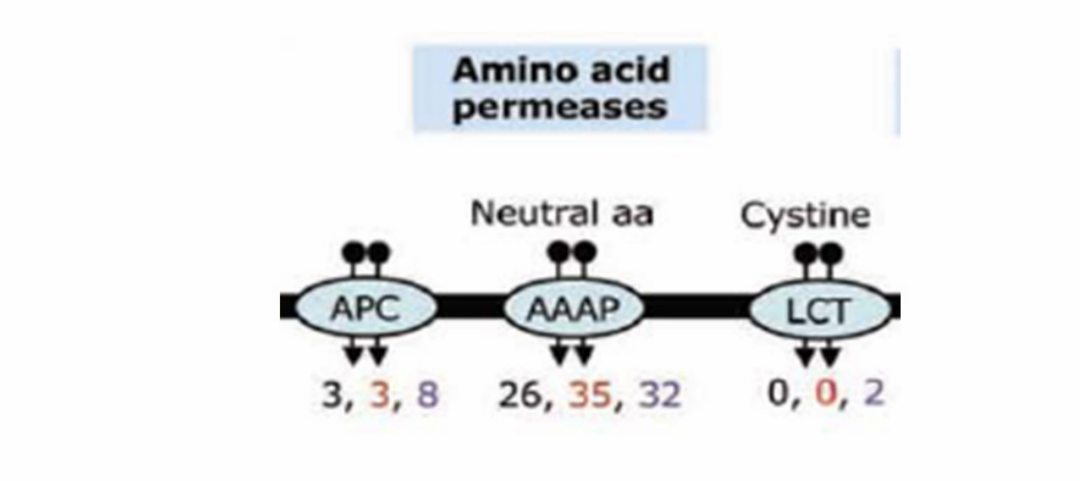


Figure 1-5: Amino acid permeases from *L. major*, *T. brucei* and *T. cruzi*, respectively (taken from Berriman *et al.*, 2005).
In *L. major* 3 belong to the amino acid/polyamine/organocation transporter superfamily (APC), 26 to the amino acid/auxin permease family (AAAP), with no lysosomal cystine transporter members present. In *T. brucei* 3 belong to the APC family, 35 to the AAAP family and no LCT family members are present. In *T. cruzi* 8 belong to the APC family, 32 to the AAAP family and 2 to the LCT family.

Large amino acid transporter gene families have been identified across the trypanosomatid species (Bouvier *et al.*, 2004; El-Sayed *et al.*, 2005; Jackson, 2007; Berriman *et al.*, 2005). Biochemical characterisation of amino acid transport has been studied but no study has linked transport to a specific genomic locus. It is clear that the genomic repertoire far exceeds biochemical characterisation, especially given the fact that transport of a particular amino acid can be shared with other structurally or functionally similar amino acids.

A systematic approach was taken to the assessment of the origin of those genes as well as associations with homolog genes in the other trypanosomatid species (Jackson, 2007). The large number of amino acid transporter genes and the fact that they are often arranged in arrays point to mechanisms by which new loci originated during evolution. It seems possible that the multitude of diverse amino acid transporter repertoires enables specialisation of individual loci to regulation of particular amino acids and enables

modulation of incoming amino acids in response to environmental changes. The study undertaken by Jackson applied a comparative approach to gene family evolution, investigating the mechanisms responsible for loci differences between species.

	Parallel Classifications	
Gene I.D.	**AATP# (from GeneDB)**	**AAT Locus**
Tb927.8.7610	AATP1	AAT7
Tb927.8.7620	AATP1	AAT7
Tb927.8.7630	AATP1	AAT7
Tb927.8.7640	AATP1	AAT7
Tb927.8.7660	AATP1	AAT7
Tb927.8.7600		AAT7
Tb927.8.7650		AAT7
Tb927.8.7670		AAT7
Tb927.8.7690		AAT7
Tb927.8.7680		AAT7
Tb927.8.7700		AAT7
Tb927.4.4830		AAT4
Tb927.4.4850		AAT4
Tb927.4.4870		AAT4
Tb927.4.4840	AATP7	AAT4
Tb927.4.4820	AATP10	AAT4
Tb927.4.4860	AATP8	AAT4
Tb927.4.3990		AAT2
Tb927.4.4000		AAT2
Tb927.4.4010		AAT2
Tb927.4.4020		AAT2
Tb927.8.8290	AATP5	AAT10
Tb927.8.8300		AAT10
Tb11.017590		AAT17
Tb11.01.7600		AAT17
Tb927.6.4660	CAATP	-
Tb927.8.5450		AAT6
Tb09.211.1760		AAT11
Tb10.6k15.0450		AAT14
Tb927.8.7740		AAT8
Tb927.4.4730	AATP11	AAT3
Tb927.4.3930		AAT1
Tb927.8.4700	AATP6	AAT5
Tb927.8.4710		AAT5
Tb927.8.4720		AAT5
Tb927.8.4730		AAT5
Tb927.8.4740		AAT5
Tb927.8.4750		AAT5
Tb11.01.7500		AAT16
Tb11.01.7520		AAT16
Tb927.8.8220		AAT9
Tb927.8.8230		AAT9
Tb927.8.8240		AAT9
Tb927.8.8250		AAT9
Tb927.8.8260		AAT9
Tb10.70.1170		AAT12
Tb11.02.4520		AAT15
Tb10.70.0300		AAT13

Table 1: Amino acid transporter genes from *T. brucei*.

Table 1 shows GeneDB identification cross-referenced to AATP number classification (Hasne & Barrett, 2000) and AAT loci (Jackson, 2007). The three genome sequences were compared and defined by their genomic position, giving specific gene loci which could be defined as shared or distinct across the tritryp genomes (Figure 1-6). Loci that are found in different genomic locations or different loci in same position point to evolutionary influences.

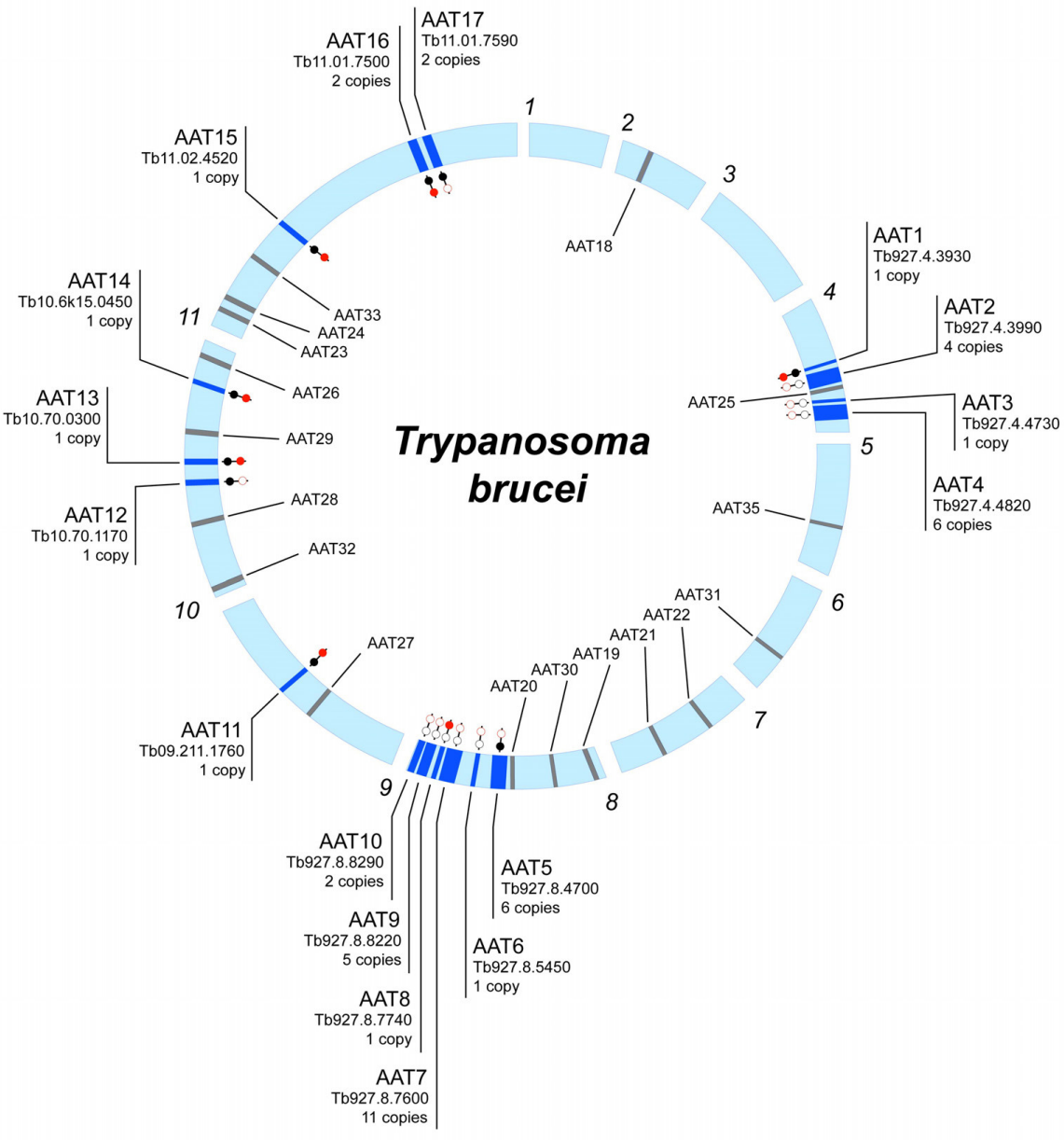


Figure 1-6: *Trypanosoma brucei* AAT loci (taken from Jackson, 2007).
11 Chromosomes are arranged circularly and labelled by number in clockwise fashion. Dark shaded bars across chromosomes represent AAT loci and are labelled with locus number, GeneDB identifier and copy number (also reflected in the band width). Grey shaded bars represent the genomic positions of AAT loci found in *L. major* or *T. cruzi*, but absent in *T. brucei*, and are labelled inside the circle. The status of each AAT locus in *L. major* and *T. cruzi* are represented by red and black circles respectively; shaded circles

indicate the presence of a homoeologous gene, open circles indicate the absence of any AAT gene, but with typically conserved synteny around the location.

This throws up an obvious problem due to the fact that the primary definition of an amino acid transporter locus is gene location, and not sequence. This presents numerous problems, for example the AATP1 locus of *T. brucei* includes an array of 11 genes but is classed as a single locus based on location (AAT7), when in reality those 11 genes group together more subtly, with 3 distinct groups emerging when comparing gene sequences (chapter 3).

Jackson concluded that the repertoires of amino acid transporter loci are fluid in both complement and gene dosage, although there seems to be a somewhat customised transporter repertoire when comparing the three species. A vacant locus in a particular species was attributed to either a loss or an origin of genomic content. However, the statement of a "customised AAT gene repertoire" is likely to translate into the unique amino acid requirements for each species but, at the same time, is unlikely to be a distinct characteristic. However, several gene loci specific to *T. brucei* (one without homeologous loci in other species) were identified (AAT2, AAT3, AAT4, AAT6, AAT7, AAT9, and AAT10).

The digenetic lifestyle of the African trypanosome, involving the mammalian bloodstream and the tsetse midgut, offers variation in temperature and nutrient availability. Developmental regulation of certain genes to adapt to divergent environments is necessary and investigations into these processes will provide more information on the cues leading up to differentiation from one life cycle stage to the next. Developmentally regulated genes in two strains of *T. brucei* have been reported using microarray analysis (Brems *et al.*, 2004). Expression patterns obtained showed that 2% of trypanosome genes showed developmental regulation at the mRNA level – including 3 groups of amino acid transporter genes upregulated in procyclic forms (Tb927.4.4730, AAT3; Tb927.8.4700, AAT5; and Tb927.4.3990, AAT2).

Differential expression of genes between the bloodstream form and procyclic form trypanosome has been investigated (Clayton, unpublished data). From this, key amino acid transporters that were upregulated in either the BSF or the PCF have been identified. In PCF trypanosomes, a single amino acid transporter Tb04.3I12.190 (Tb927.4.4730, AATP11, AAT3) has been shown to be upregulated. Regulation of TbAATP11 mRNA has

been investigated (Robles & Clayton, 2007), showing that the 3'-UTR causes higher expression of the reporter gene in PCF trypanosomes, with multiple elements within the intergenic sequence being required for this control. The mRNA of this particular amino acid transporter seems to be under the control of the 5'-3' exonuclease XRNA and shows a half-life of 10 mins and 60 mins in BSF and PCF trypanosomes respectively (Robles & Clayton, 2007).

In BSF trypanosomes, two amino acid transporters, Tb04.3I12.30 (Tb927.4.4820, AAT4, AATP7/8/10) and Tb04.3M17.60 (Tb927.4.4870, AAT7, AATP1) are shown to be upregulated. Tb927.4.4730 is closely related to Tb927.8.7740, showing greater than 90% similarity. Tb927.4.4820 sits at the start of a six gene array comprising of Tb927.4.4820, 4830, 4840, 4850, 4860 and 4870. At this point in time we are unable to assign a phenotype to those amino acid transporters that are differentially upregulated. However, by analysing the amino acid requirements of both forms of the parasite we can infer metabolic consumption of amino acids and relate that to amino acid transporters (chapter 3).

1.18 Drug Uptake via Amino Acid Transporters

Amino acids are important for protozoa as energy sources as well as forming pools of soluble osmolites (Burchmore *et al.*, 2002). Selective plasma membrane transporters can be exploited in the delivery of drug targeting. Amino acid transporters are one group of transporters that fulfil the criteria needed for successful exploitation of nutrient transport systems for drug delivery (chapter 1.11).

The precedent set by work with the P2 transporter, with regard to targeting toxins to the trypanosome interior via nutrient transporters, inspired a search for amino acid analogues with trypanocidal activity that might enter trypanosomes through members of the amino acid transporter family. Several amino acid analogues that kill trypanosomes are known. Most notably, Acivicin and 6-diazo-5-oxo-l-norleucine (DON), which are glutamine analogues and inhibit CTP synthetase and can kill trypanosomes *in vitro*. Trypanosomes have very low levels of CTP due to limited capacity for *de novo* synthesis and lack of salvage pathways for cytidine (Hofer *et al.*, 2001). Acivicin and DON reduce CTP levels even further, inhibiting parasite growth *in vitro*. The similarity between trypanosomiasis and cancer (Barrett & Barrett, 2000) is further exemplified by the fact that the pharmacological properties of acivicin and DON have been extensively studied as cancer drugs. However, these glutamine analogues only arrest the proliferation of the parasites. A functional immune response is then required to eradicate the static trypanosomes (Fijolek

et al., 2007). Most recently, the methionine analogue buthionine sulphoxamine (BSO) (Huynh TT *et al.*, 2003; Arrick *et al.*, 1981) has been identified as a potential trypanocide. The mode of action of BSO seems to involve inhibition of γ-Glutamylcysteine synthetase (γ-GCS) resulting in trypanolytic effects. However, γ-GCS inhibition was not conclusively shown to be the sole target for BSO.

1.19 Trypanosomes & the Blood-Brain Barrier

The neurological manifestations of "sleeping sickness" in man are attributed to the penetration of the blood-brain barrier and invasion of the central nervous system by *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*. However, how African trypanosomes cross the blood-brain barrier remains an unresolved issue. Recent progress in understanding the neuropathological mechanisms of sleeping sickness reveals a complex relationship between the parasite and the host nervous system (Engana *et al.*, 2002; Mulenga *et al.*, 2001).

Following the insect bite, parasites replicate at the site of infection, producing a local inflammatory reaction (Winterbottom's sign), then spread to the lymph nodes and eventually throughout the entire blood stream with final parasite establishment in the CNS. This is known as late stage HAT, or sleeping sickness, and it compromises the integrity of the blood-brain barrier due to the inflammation that occurs when parasites become associated with the brain capillaries. It is as yet unknown how trypanosomes penetrate the blood-brain barrier but it is thought that the general permeabilisation of the barrier during the accumulation of mononuclear cells to the site of infection could influence the migration of trypanosomes (Engana *et al.*, 2002). Moreover, it has been shown that IFN-γ is an essential cytokine in allowing trypanosomes to circumvent the BBB (Masocha *et al.*, 2004). There is also evidence that parasites can cross the BBB without a generalised loss of tight junction proteins (Mulenga *et al.*, 2001), suggesting migration of parasites by transcytosis through the cerebral endothelial cells rather than between them.

As well as multiple systemic features seen in the early acute stage, the late chronic stage is associated with a wide range of neurological features; including neuropsychiatric, motor and sensory abnormalities, all of which may not occur in the same patient. Late stage trypanosomiasis is often gradual in onset and differs depending on the *gambiense* or *rhodesiense* disease (Kennedy, 2006). The CNS features observed in trypanosomiasis stretch further than the characteristic sleep disturbances which give the disease its name.

The features can broadly be divided into psychiatric disturbances, sleep disorders, motor system disorders, sensory syndromes and abnormal reflexes (Kennedy, 2006). In the final stages of the disease the sufferer develops seizures, incontinence, cerebral oedema, progressive mental deterioration, and finally death as a direct result of the disease or indirect result such as intercurrent infection or heart failure.

Psychiatric disturbances of the late stage CNS involvement of African trypanosomiasis include, but are not limited to, anxiety and irritability, lassitude and indifference, mania and agitation, violent and suicidal behaviour, uncontrolled sexual impulses, hallucinations and delirium. Sleep disturbances include daytime somnolence, nocturnal insomnia, narcoleptic crises, and uncontrollable urges to sleep. Motor disturbances include limb and tongue tremors, muscle fasciculation, slurred speech and cerebella ataxia, focal motor weakness, neuritis and polyneuritis. Sensory involvement includes parathesia and pruritis.

How the parasite perturbs brain function to bring about the various CNS features is a problem that is not well understood and involves many possible immunopathological pathways. Many abnormalities of CNS-involved trypanosomiasis have been reported (Kennedy, 2004), some of which include a diffuse perivascular infiltration with lymphocytes, macrophages, and activated astrocytes. It seems evident from studies that early astrocyte activation plays an essential role in driving the inflammatory response (Kennedy *et al.*, 1997). Current understanding of parasite neuropathogenesis is based upon the balance between pro-inflammatory and counter-inflammatory cytokines, macrophage activation by cytokines and parasite components, and interactions between cytokines and chemokines (Kennedy, 2006).

Any potential trypanocidal drug for late stage disease will need to cross the BBB. Entry into the CSF by potential drug candidates is by three main routes; passive diffusion of lipid-soluble compounds, trans-cytosis, and by transport of specific water-soluble substances using transporters (Engana *et al.*, 2002). The brain is one of the least accessible organs for the delivery of active pharmacological compounds due to the fact it creates a unique physical and enzymatic barrier, capable of metabolising nutrients and drugs. BBB capillary endothelia lack fenestrations, are sealed by tight junctions and contain specific efflux transporters for nutrients, drugs and waste products (Witt & Davis, 2006).

The influence of brain pathology during disease must be taken into account when developing drug delivery strategies. Several disease states have been reported to increase BBB permeability to fluid and solutes (Banks *et al.*, 1996) and trypanosomiasis is one of

them. Increasing bioavailability to the CNS can be achieved via different methods depending on the class of drug in question, including pharmacological-based approaches to increasing specific biochemical properties and physiological-based approaches to exploit the various features and characteristics of the BBB. One such example of the latter is exploiting nutrient transporters of the BBB. Levodopa (L-Dopa), a lipid-insoluble compound used for the treatment of Parkinson's disease, is the prime example. L-Dopa availability within the CNS is very high due to the fact that it is actively transported across the BBB by the large neutral amino acid carrier, System L (Wade & Katzman, 1975). Uptake has been shown to be time-dependent, temperature-sensitive, and Na^+-independent (Kageyama *et al.*, 2000).

There is evidence that changes in the concentrations of the monoamine neurotransmitters within the brain are associated with changes in mental processes, with disorders of control of movement and with certain neuropsychiatric diseases (Daniel *et al.*, 1976). These neurotransmitters are synthesized in the brain from aromatic amino acid precursors that have to be obtained from the circulating blood. Changes in the cerebral concentrations of one or more neurotransmitters are the cause of certain neuropsychiatric disorders (Fernstrom & Wurtman, 1971; Shoemaker & Wurtman, 1971; Bernheimer & Hornykiewicz, 1973). Moreover, other amino acids like the branched-chain amino acids (leucine, isoleucine and valine) are involved directly and indirectly in a variety of important biochemical functions in the brain (Fernstrom, 2005). The branched-chain amino acids influence brain function by modifying transport of aromatic amino acids into the CNS. Transporter competition between the branched-chain amino acids and the aromatic amino acids mean that a rise in plasma branched-chain amino acid concentration leads to decrease in aromatic amino acid availability within the CNS. The formation of neurotransmitters is largely determined by how the CNS can acquire pre-cursor aromatic amino acids from the circulating blood (Coppen *et al.*, 1963; Daniel *et al.*, 1976). More specifically, elevating brain tyrosine concentrations stimulates catecholamine production (Fernstrom & Fernstrom, 2007).

Trypanosome-induced alterations and deprivations to host environment is wide ranging with trypanosomiasis. Evidently, trypanosome tryptophan metabolism is thought to contribute to the pathogenesis of trypanosomiasis (Vincendeu *et al.*, 1999). The aromatic amino acids are involved in transamination reactions of α-ketomethiobutyrate and methionine recycling in trypanosomes (Berriman *et al.*, 2005; Berger *et al.*, 1996; El Sawalhy *et al.*, 1995; Vincendeau *et al.*, 1999). It has been noted that a significant decrease in serum tryptophan levels during trypanosomiasis occurrs regardless of late stage

involvement (Vincendeau *et al.*, 1999). Studies investigating the importance of tryptophan metabolism pointed to an essential role for tryptophan in parasite growth both in an *in vivo* and *in vitro* setting (Vincendeau *et al.*, 1999). However, the aromatic amino acids do not serve as a carbon source for growth. Moreover, decreased levels of the neurotransmitter serotonin within the brain may be one of the pathophysiological mechanisms provoking sleeping sickness. The functional significance of tryptophan catabolism is unclear. However, it is important to note that the range of pathophysiological effects associated with the disease are linked to trypanosome involvement, possibly by reducing host levels of essential amino acids (El Sawalhy *et al.*, 1995). Another possibility is the direct involvement of toxic or inhibitory actions of metabolites produced from aromatic amino acid metabolism in trypanosomes. The aromatic α-keto acids and indole-3-pyruvic acids have been shown to accumulate abnormally in the urine of infected mice (El Sawalhy *et al.*, 1995). This pathological symptom has been likened to other inherited disorders of aromatic amino acid metabolism. Indeed, the correlation between the physiological manifestations of trypanosomiasis and other neurological disorders cannot be discounted. It is evident that the perturbations of host environment brought about by trypanosome amino acid metabolism result in pathogenesis and physiology of the disease.

2 Materials & Methods

2.1 Cell Culture

2.1.1 Trypanosomes

T. brucei procyclic form (PCF) strains 427, 927, and various derivatives were cultivated in SDM79 medium (Brun & Schonenberger, 1979), Cunningham's medium (Cunningham, 1972) and SDM80 medium (Appendix 8.2) supplemented with 10% (v/v) heat inactivated FCS (for SDM80 dialysed against 0.15 M NaCl with a molecular mass cut-off of 10,000 Da), at 25-28˚C. A typical culture was started at 10^5 cells/ml. After 4 days, the culture reached mid-log phase (10^6 cells/ml) and after 7 days the stationary phase was reached (10^7 cells/ml).

T. brucei bloodstream form (BSF) strain 427 were cultured in HMI-9 medium containing 10% heat inactivated FCS (Hirumi *et al.*, 1977; Hirumi, 1994) at 37˚C in a 5% CO_2 incubator.

T. brucei PCF strain 29-13 (La Count *et al.*, 2000) were used in all RNAi experiments. For the transgenic trypanosomes, the tetracycline repressor constructs were maintained by the addition of 15 µg/ml G418 and 25 µg/ml Hygromycin B to the medium. To induce the RNAi effect, tetracycline was added at a concentration of 100 ng/ml.

Development of cultures was monitored by microscopy and cell numbers were determined using an improved Neubauer haemocytometer (counting chamber; Weber Scientific).

2.1.2 Mammalian cells

The Human Embryonic Kidney cell line strain 293T (HEK) were cultured in Dulbecco's Modified Eagle's Medium (Sigma) with penicillin/streptomycin (10 mg/ml), L-Glutamax (200 mM), and 10% FCS in vented culture flasks at 37ºC in 5-10% CO_2 atmosphere, passaging when cells on the monolayer were 80-85% confluent. Cells were passaged by the addition of pre-warmed 0.25% Trypsin-EDTA solution (Sigma). After detachment of cells from the culture dish surface, cells were mixed with a further 8 ml of pre-warmed medium and spun down at 1,200 rpm for 5 mins. Supernatant was decanted and pelleted cells were resuspended in 10 ml of medium, with 1 ml of cell suspension added to a fresh sterile culture dish containing 10 ml of medium.

Development of cultures was monitored by microscopy and cell numbers were determined using an improved Neubauer haemocytometer (counting chamber; Weber Scientific).

2.2 Alamar Blue Assay

The Alamar blue assay is used to determine the relative number of live cells in a population (Raz *et al.*, 1997). The assay was employed to test potential trypanocidal drugs or as a measure of growth by different carbon sources. The assay was carried out on both PCF and BSF trypanosomes.

Trypanosomes (typically at a density of 10^5 per 100 µl for PCF and 10^4 per 100 µl for BSF) were incubated for 72 hrs in 96-well flat bottomed microtiter plates. Drug concentration started at 100 µM and decreased in doubling dilution across rows in the plate, with no drug used as a control. All experiments were performed twice with each drug in duplicate.

Alamar blue reagent (Resazurin; Raz *et al.*, 1997), at 12.5 mg per 100 ml, stored and protected from light at 4ºC, was added after 48 hrs incubation (at 10% - 20 µl per well). Measurement of fluorescence was carried out in a Millipore Cytoflour plate reader (Perkin Elmer, LS 55, Luminescence Spectrometer) at 530 nm excitation wavelength and 590 nm emission wavelength after a further 24 h incubation. The IC_{50} value is the concentration inhibiting a parameter (growth in this case, or conversion of oxidised to reduced resazurin) by 50%.

Human Embryonic Kidney cells (strain 293T) were used as the mammalian cell line for comparative analyses and to give a preliminary therapeutic index. The Alamar blue assay protocol was modified from the one used for live trypanosomes. Briefly, 100 µl of a 3 x 10^5 cells/ml suspension was added to each well of a 96-well plate and incubated at 37ºC for 3 hours to allow cells to adhere to the bottom of the wells. Preparation of drug stocks in doubling dilution was added after the incubation period, incubated for a further 12-16 hours before the addition of 10% resazurin. After 24 hours plates were read.

In all, 92 amino acid analogues (Appendix 8.14, chapter 6) have been screened for trypanocidal activity using the Alamar Blue method.

2.3 Isolation of genomic DNA

For isolation of genomic DNA from *T. brucei*, 10^8 parasites were pelleted by centrifugation at 2,000 g for 10 min at 4˚C and resuspended in 150 µl of TELT buffer (Appendix) (Medina-Acosta & Cross, 1993) and incubated at room temperature for 5 min.

150 µl of Phenol-Chloroform was added and mixed gently by inversion. The aqueous mix was centrifuged at 13,000 g for 5 mins at room temperature. The aqueous layer was transferred into a sterile 1.5 ml Eppendorf tube and the DNA was precipitated by addition of 1/10 volume of 3 M sodium acetate and 2 volumes of 100% ethanol and placed on ice for 5 mins. The DNA was pelleted by centrifugation at 13,000 g at room temperature. The pellet was washed in 1 ml 70% ethanol, air dried for 5 mins and resuspended in TE buffer (Appendix 8.6). The DNA was incubated with 1 mg/ml RNAse (Sigma) for 30 min at room temperature and stored at -20˚C for further work.

2.4 Total RNA Isolation

A pellet of 10^8 cells was resuspended in 1 ml of Trizol reagent (Gibco), comprising monophasic solutions of phenol and guanidine isothiocyanate, to maintain the integrity of RNA (Sambrook & Russell, 2001). The mixture was incubated at room temperature for 5 mins before 200 µl of chloroform was added, then mixed by inversion. Organic and aqueous phases were separated after a further 2 min of incubation at room temperature. Addition of chloroform generates an organic phase into which DNA and proteins are extracted, leaving RNA in the aqueous supernatant. After centrifugation for 15 min at 12,000 g and 4˚C, the aqueous phase was transferred to a fresh sterile 1.5 ml Eppendorf tube and 0.5 ml of isopropyl alcohol was added. The mixture was incubated for 10 min at room temperature to precipitate the RNA. The RNA pellet was collected by centrifugation at 12,000 g for 10 min at 4˚C, and then washed with 70% ethanol, air dried for 10 min and resuspended in RNAse-free water. RNA was then stored at -20˚C.

RNA is chemically more active than DNA; ergo it is prone to digestion by contaminating RNases. The simple and most effective way to guard against RNase contamination is prevention. Sterile technique and proper sterile equipment were used throughout. RNases are ubiquitous, resistant to prolonged boiling, and are able to refold after denaturation (Sambrook & Russell, 2001). All equipment and reagents were made RNAse free by treatment with 0.01% diethylpyrocarbonate (DEPC). DEPC is a highly reactive alkylating agent that destroys the enzymatic activity of RNase.

2.5 Molecular Cloning Techniques

2.5.1 Polymerase Chain Reaction (PCR)

PCR was used to amplify segments of DNA between two known regions for various cloning exercises. All oligonucleotides were synthesized by MWG-Biotech. The two primers (forward and reverse) used in each amplification had sequences complementary to those flanking the region for amplification. One primer was designed in the 5'-3' direction of the sense strand (forward primer) and the other was designed in the 5'-3' direction of the anti-sense strand (reverse primer) of DNA. Enzymatic replication of DNA used *Taq* DNA polymerase (Promega). A 50 µl reaction contained 200 µM of dNTP, 50 µl of 10 X buffer, upstream and downstream primers at 0.1 µM, 200 ng of genomic DNA and 1 unit of *Taq* DNA Polymerase. The amplification was performed using a thermal cycler (PTC200 DNA Engine Thermal Cycler, MJ Research) under differing conditions. Temperature and incubation times were optimized for each reaction. The PCR was performed in three stages: 94˚C for 2 min followed by 30 cycles composed of a denaturation step at 94°C for 15 sec, an annealing step at T_m for 30 sec and an amplification step at 72°C for 2 minutes, a final extension at 72°C for 5 minutes. The T_m is the annealing temperature, which was chosen to be a few degrees below the predicted T_m of each primer pair. Correctly sized products were isolated by electrophoresis on a 1% agarose gel containing ethidium bromide and then purified using a QIAquick PCR purification kit according to manufacturer's instructions (Qiagen).

2.5.2 Plasmid Vectors

DNA fragments can be inserted into vectors and efficient cloning is achieved when DNA insert and plasmid are digested by restriction enzymes creating "sticky" ends. TA cloning takes advantage of the single A-residue at the 3'end of the insert generated during PCR by *Taq* DNA polymerase enzyme. The specific vector pGEM-T (Promega) contains a single T-residue overhang allowing ease of cloning.

PCR fragments were cloned into the pGEM-T vector. Ligation reactions were carried out using a 3 fold-excess in terms of molar ends of the insert DNA and the vector. The ligation reaction was performed under the following conditions: final concentration of 1X ligation buffer, 1 µl vector (20-50 ng/µl), 3 µl PCR product, 1 µl T4 DNA Ligase (1 U/µl). The reaction was incubated for at 16˚C for at lest 5 hrs. A positive control (control insert DNA) and a background control (digested vector without insert DNA) were used to assess re-

ligation of the vector. Once plasmid had been purified and the presence of insert verified the PCR products could then be digested, isolated and cloned into various other vectors depending on use (see relevant chapters).

Restriction enzymes recognize specific palindromic sequences in the DNA. Standard restriction enzyme digests were typically performed using a final concentration of 1X restriction enzyme buffer, 50-100 ng of DNA, 1 unit BSA, and 5 units of enzyme. The digest mixtures were incubated at 37°C for at least 2 hrs or until complete digestion was obtained.

2.5.3 E.coli Transformations

The *E. coli* chemically competent cell line JM109 (Promega) were transfected with plasmid containing the DNA segment of choice. 50 µl of cells were added to a chilled Eppendorf tube containing 10 µl of ligation mix. The tubes were left on ice for 30 min, then heat shocked for 3 min at 42°C and immediately placed on ice for 1 min and spread on LB agar plates containing ampicillin (100 µg/ml), isopropylthio-β-D-galactoside (IPTG; 40 µg/ml), and 5-bromo-4-chloro-indolyl-β-D-galactoside (X-Gal; 40 µg/ml). IPTG and X-Gal were added for blue/white colony screening. Successful cloning of the insert into pGEM-T was indicated by the presence of white colonies as opposed to blue colonies.

Many commonly used plasmid vectors carry a short segment of DNA containing the regulatory sequence and the coding information for the first 146 amino acids of ß-galactosidase (*lacZ*). Usually, host cells express the complementary C-terminal portion of ß-galactosidase, and together host and transformed vector will express the active enzyme. This is known as α-complementation. Insertion of a fragment of foreign DNA results in the disruption of the *lacZ* gene, the resulting ß-galactosidase transcript is non-functional and α-complementation is abolished. Bacteria that result from α-complementation form blue colonies in the presence of the chromogenic substrate X-Gal. Therefore colonies containing recombinant plasmids appear white. IPTG is an analogue of lactose that inactivates the *lacZ* repressor and therefore induces transcription of the *lac* operon. The fact that IPTG is nonfermentable means it can act as a gratuitous inducer of the *lac* operon.

For other vectors that do not allow blue/white screening, insertion of correct insert was confirmed by PCR. Briefly, a PCR reaction was set up broadly as previously described, containing primers specific for the insert. The DNA template, however, was simply a single bacterial colony picked from a plate and dipped into the PCR reaction mix (with the

colony preserved as a culture). Generation of a PCR product indicated successful insertion of the gene target into the plasmid vector.

For each transformation, single transformed colonies (containing vector and insert) were picked from plates into 5 ml of LB/ampicillin (100 µg/ml) medium and incubated overnight at 37°C under vigorous shaking. The bacterial cells were harvested by centrifugation at 13,000 rpm for 15 minutes at 4°C and the pellets processed using a Qiagen Plasmid Miniprep Kit according to the manufacturer's specification. The plasmid DNA quality was analyzed by agarose gel electrophoresis after the liberation of the inserted fragment from the vector by restriction enzyme digestion as previously described. Purified but non digested DNA plasmid was used as control. The vectors with cloned inserts were sent to MWG-Biotech for sequencing.

For the preparation of large-scale plasmid DNA, a pre-culture of 5 ml, with a positive transformed colony, was grown overnight and then transferred to 100 ml of LB/ampicillin medium, which was grown overnight at 37°C under shaking. The culture was spun and the bacterial pellet processed using a Qiagen Plasmid Maxiprep Kit according to the manufacturer's specification. The yield and quality of the plasmid DNA was also assessed by the NanoDrop® ND-1000 UV-Vis Spectrophotometer.

2.6 Northern Blot Analysis

Northern hybridization is used to measure the amount and size of RNAs transcribed from a particular gene. RNA samples were separated according to size using 1% agarose/formaldehyde gel electrophoresis for at least 4 hr at 70 V. The gel contained 1% agarose, 12% formaldehyde, and 2 X northern gel buffer (1 X MOPS, Appendix 8.10). RNA samples were prepared by adding the RNA (4 µg) to RNA loading mix consisting of 12.5% formaldehyde, 50% formamide and sterile water to 20 µl. Formamide is used to assist the denaturation of RNA before electrophoresis through the gel. RNA mix was then heated briefly at 65˚C for 15 min. RNA was transferred to a nylon membrane (Hybond N, Amersham) by capillary transfer under 20 X SSC – a technique similar to that used in Southern blotting (Sambrook & Russell, 2001), that involves transfer of nucleic acids from gel to membrane in an upward flow of buffer. After transfer the RNA was UV cross-linked to the membrane, using a Stratalinker apparatus set at "optimal cross-link." The membrane was then air dried and is now ready for hybridisation, and can be stored in a sealed plastic bag in the dark at room temperature indefinitely.

2.7 Probe Labelling

Prehybridisation of the membrane was carried out for a minimum of 2 hr at 65˚C in 50% formamide, 6 X SSC, 5 X Denhardt's solution, 1% SDS, and 0.1 mg/ml denatured sonicated salmon sperm DNA. Purified PCR product was labelled using α^{32}P (Amersham). Random priming of PCR product was done using the Prime It II Kit (Stratagene). Briefly, 10 µl of random oligonucleotide primers were added to 25 ng of DNA in a total reaction volume of 34 µl. The mixture was denatured by boiling for 5 min and then snap-cooled on ice. 10 µl of 5 X dATP primer buffer, 50 µCi α^{32}dATP and 1 µl of Exo-Klenow enzyme (5 U/µl; Stratagene) were added and incubated at 37˚C for 10 min. The Exo-Klenow Fragment is the large fragment of DNA Polymerase I (obtained from *E. coli*). It exhibits 5'-3' polymerase activity, but lacks the 3'-5' and 5'-3' exonuclease activities of DNA Polymerase I. The labelled probe was finally denatured by boiling for 5 min before addition to the membrane and hybridization buffer. Hybridization was performed overnight at 42˚C in the same prehybridisation solution lacking Denhardt's solution but containing 50 % formamide and the denatured probe. After overnight hybridization, blots were washed once in 10 X SSC, 1% SDS for 30 min at room temperature, once in prewarmed 1 X SSC, 0.5 % SDS for 45 min at 42˚C, and once in prewarmed 0.1 X SSC, 0.2 % SDS for 30 min at 42ºC. Blots were then dried, covered with a sheet of saran wrap and exposed to X-ray film for at least 16 h at -80ºC then developed using Compact X4 X-ograph (Imaging systems).

Nylon membranes containing either genomic DNA or RNA can be stripped and rehybridised up to 4 times. Hybridized probes can be stripped by immersing the membrane in boiling SDS solution (0.1%), repeated twice with a fresh batch of boiling solution. Membrane was then dried and prehybridised before hybridization with a second probe.

2.8 RNA Interference in T. brucei

RNA interference (RNAi) refers to the introduction of homologous double stranded RNA (dsRNA) to specifically target a gene product of interest, resulting in an apparent "null" phenotype caused by the ablation of mRNA (chapter 1.9).

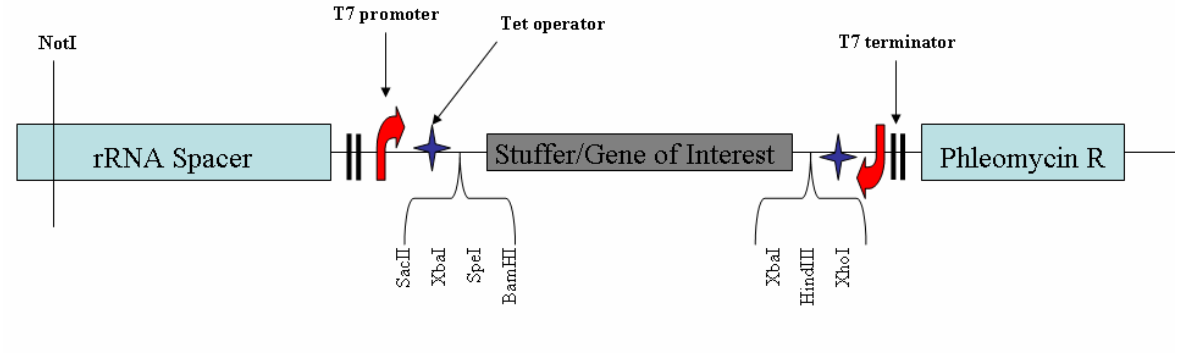


Figure 2-1: RNAi vector p2T7Ti.

The RNAi vector (Figure 2-1) used in this study (p2T7Ti) is based on the strategy of placing the sequence of interest between opposing T7 promoters (La Count *et al.*, 2000). The vectors contain a rRNA spacer for integration into the rRNA locus of the parasite's genome, a tetracycline inducible operator, as well as a drug resistance gene for selection (phleomycin). The vector must be used in specially derived *T. brucei* cell lines (29-13), which express bacteriophage T7 RNA polymerase and the tet repressor from bacteriophage λ. The T7 polymerase and the tetracycline repressor constructs were maintained in the 29-13 cell line (La Count *et al.*, 2000) by the addition of 15 µg/ml G418 and 25 µg/ml Hygromycin B to the medium. To induce the RNAi effect, tetracycline was added at a concentration of 100 ng/ml. Without tetracycline, the tet repressor expressed in the cells binds to the tet operator to inhibit transcription from the integrated construct. With the addition of tetracycline, the repressor is bound and its action prevents binding to the operator allowing transcription to occur.

2.9 Plasmid Construction

For the RNAi constructs, gene fragments of around 400-600 bp in size were amplified by PCR of gDNA using specific primers containing appropriate restriction enzyme linkers. Isolated, purified PCR products were cloned firstly into the pGEM-T vector following a cloning strategy as previously described. Following digestion and isolation from the pGEM-T vector, the PCR generated fragments were cloned into the appropriate RNAi vector, transfected into competent *E. coli* bacteria and positive transformed colonies grown up in large quantities. Plasmid DNA was extracted ready for transfection into the parasite.

2.10 Transfection of Trypanosomes

For stable transfection of the procyclic host strain 29-13 via integration into an rDNA spacer region, the RNAi constructs were linearised by *NotI* digestion (Wirtz, 1995). The digestion of plasmid DNA was carried out in a volume of 40 µl using 1 unit of the enzyme, 4 µl of 10X buffer and 10 µg of plasmid DNA and incubated at 37°C for two hours. After the addition of 1 unit of the enzyme, the reaction was re-incubated overnight, with the final digest checked by agarose gel electrophoresis.

The linearised DNA plasmid was sterilized, by ethanol precipitation after an inactivation of the enzyme at 65°C for 20 minutes, then air dried in a sterile hood and resuspended in 20 µl of sterile water. Agarose gel electrophoresis was used to assess proper linearization of the vector.

For the transfection, PCF 29-13 cells were grown to mid-log phase (5 x 10^6 cells/ml). 5 x 10^7 cells were collected by centrifugation at 1,500g for 10 min at room temperature, washed twice in 10 ml of ZPFM buffer (Appendix 8.11) and then resuspended in 0.5 ml of the buffer. To minimise the possibility of degradation, linearised plasmid DNA was added to the cells just before electroporation. Cells were electroporated by using a Biorad Gene Pulser set at 1.4 kV and 25 µF in 0.4-cm-gap cuvettes using two pulses delivered 10 seconds apart. Immediately following transfection, cells were transferred into 10 ml of fresh pre-warmed SDM79 medium with 10% heat inactivated foetal calf serum supplemented with neomycin, hygromycin. Drug selection was started after overnight recovery to allow the build up of resistant proteins.

After one day the 10 ml culture of cells was split into the top 6 wells of a 24-well microtitre plate, and diluted down stepwise by doubling dilution. Drug resistant cells were selected between 7 to 14 days and then cloned by limiting dilution in 96-well plates.

2.11 Analysis of Growth Rates

Growth rate analysis was also used to assess the effect of trypanocidal drugs on BSF trypanosomes, growth of RNAi cell lines upon tetracycline induction, and the effect of different carbon sources for growth of PCF trypanosomes. PCF cultures were commonly initiated at a starting density of 5 x 10^5 cells/ml. For RNAi cell lines growth was determined in the presence and absence of tetracycline. Cells were grown in 24 well plates, using a total volume of 1 ml of SDM79 medium, CM or SDM80 medium (Appendix 8.2)

supplemented with proline or glucose individually at 10 mM (other supplements were added at concentration specified in the results section).

In general, master cultures were propagated in SDM79 (strain 427) or CM (strain 927). Differing medium conditions employed a general strategy of centrifugation of aliquots of the master cultures (1,250 rcf for 10 mins) and resuspending cells to the desired cell density. Experimental counts involving SDM80 based medium (with or without supplementation) involved an overnight recovery in SDM80.

BSF cultures were typically started at a density of 2 x 10^4 cells/ml, in HMI-9 medium in the presence and absence of drug. Parasite numbers were measured by counting with an improved Neubauer haemocytometer (Weber Scientific).

2.12 Radiolabeled Uptake Assays

Parasites were harvested during the mid-log phase of growth, at indicated times post-induction, by centrifugation at 1,250 rcf (2,500 rpm) for 10 minutes and washed three times with assay buffer (CBSS or PBS; Appendix 8.3, 8.12) by centrifugation at 1,250 rpm at 4ºC. Parasites were resuspended in assay buffer at the density of 2 x 10^8 cells/ml and kept on ice, and brought up to room temperature when the experiment was about to proceed.

Uptake of radiolabelled compounds was determined using a derivation of the rapid oil/stop spin protocol, as previously described (Carter & Fairlamb, 1993). Transport was initiated with 100 µl of cells being mixed with 100 µl assay buffer, containing radiolabelled compounds (Amersham), at a specific concentration of (see relevant results section) plus or minus other test compounds, at the concentrations specified in the Results sections. Uptake was left to proceed over time points specified in the results section.

To separate trypanosome cells from radiolabelled amino acid solution, oil was used (1-Bromodo-decane, density: 1.066 gcm^{-3}). The buffer was layered over a 90 µl cushion of oil. The oil's density, upon centrifugation, separates the trypanosome cells from the radioactive medium containing labelled amino acid. The tube was flash frozen in liquid nitrogen, and the pellet was separated from the remnants of the tube with a tube cutter, and lysed in 200 µl SDS (2%) and mixed with 3 ml scintillation fluid (Ecoscint A, National Diagnostics) and incorporated activity counted after 24 hrs (to avoid luminescence) using a liquid scintillation counter (Perkin Elmer, liquid scintillation & luminescence counter,

1450 Microbeta). All experiments (at each time point for each substrate) were carried out, at least, in duplicate, and three times independently.

Non-transported radiolabeled compounds, associated with cells or in interstitial spaces, were measured by performing control uptake determinations on ice (by commencing the reaction at a zero time point using cells, and buffer kept on ice to ensure, essentially, no uptake).

2.13 Metabolomic Profiling

Metabolomics requires special approaches for sample preparation, separation, and mass spectrometric analysis (Dettmer *et al.*, 2007). Rapid quenching of metabolism with hot ethanol has proven to be an effective method of identifying metabolites (D. Wildridge, personal communication). It is necessary to also analyse culture medium without cells in order to generate spectra of medium associated metabolites to subtract from the metabolic profile. Sample preparation involves bringing the large quantity of cells grown into a format that is compatible with the analytical technique used, while removing components (serum proteins) that will interfere with the analysis (Dettmer *et al.*, 2007; M Kamleh, personal communication). The procedure includes a pre-concentration step to achieve the detection limits required. The large volume of cells grown are pelleted by centrifugation at 1,250 rpm at 4ºC and resuspended at a concentration of 2 x 10^8 cells/ml in serum-free medium and incubated at room temperature for 30 mins. Cells were separated from medium by spinning down at 6,000 rpm for 5 mins at 4ºC. 0.75 ml of 80% EtOH/20 mM HEPES (pH 7) at 80ºC was quickly added to separated medium and cell pellet, cooled on ice for 5 mins, vortexed, and then the supernatant recovered by centrifugation at 13,000 rpm for 5 mins. Recovered supernatant was frozen in liquid nitrogen and stored at -80ºC for further analysis.

Samples were processed using an Orbitrap Mass Spectrometer (Kamleh M & Watson D, University of Strathclyde). Metabolites were separated on a ZIC-HILIC (Sequant) chromatographic column over a period of 55 minutes by eluting 10 µl of sample with 80% Acetonitrile/20% water (0.1% formic acid), gradually increasing the concentration of the aqueous component to 60%, and then returning to 20%. The Orbitrap mass spectrometry, employed here, is not strictly quantitative (in the absence of an authenticated standard). However, the data generated is quantitative in a relative sense. The increase or decrease in size of a given peak is related to the changes in abundance of that metabolite across conditions. The exact masses derived from the Orbitrap mass spectrum are assigned

putative formulae using an algorithm that sums all possible combinations of the elements C, H, O, P, N, S. Although for a given mass there may be many possible elemental combinations, in practice many of these can be eliminated as improbable.

2.14 Bioinformatic Analyses

The data was analysed using the GraFit 5.0 data analysis software (Erithacus). Kinetic constants were determined by non-linear regression analysis using the Michaelis-Menten equation. Vector NTI was used to analyse DNA and protein sequences. Sequence alignments were studied using Clustal W (www.ebi.ac.uk). Clustal W is a general purpose multiple sequence alignment program for DNA or proteins, which calculates the best match for the selected sequences, and lines them up so that the identities, similarities and differences can be seen, and apparent evolutionary relationships can be seen via viewing Cladograms or Phylograms. Prediction of transmembrane domains within amino acid sequences was done by TMpred (www.ch.embnet.org). The TMpred program makes predictions on membrane-spanning regions and their orientation. The following web resources were used to analyse nucleic acid and amino acid sequences. Metabolomic data was analysed using the Cytoscape software (chapter 5).

Databases:

TIGR database: www.tigr.org

NCBI database: www.ncbi.nlm.nih.gov

GeneDB: www.genedb.org

KEGG: www.genome.jp/kegg

3 RNAi Knock-down Approaches to Characterising Candidate Amino Acid Transporter Genes in *Trypanosoma brucei*

3.1 Introduction

3.1.1 Amino Acid Usage in Trypanosomes

The completion of the *T. brucei* genome sequence (Berriman *et al.*, 2005) highlighted several interesting characteristics of trypanosome amino acid metabolism. It revealed that most of the enzymes of the classical pathways for aromatic amino acid oxidation are missing. Conversion of phenylalanine to tyrosine does not occur in *T. brucei*, due to the fact that it does not have the enzyme phenylalanine-4-hydroxylase. All trypanosome species have genes for transamination and reduction to the corresponding aromatic lactate derivative. Branched chain amino acids can be converted to acetyl-CoA derivatives within the mitochondria. In *T. brucei* threonine is apparently not oxidised via the 2-oxobutyrate pathway, instead being degraded to acetyl-CoA and glycine by a mitochondrial specific threonine dehydrogenase. Catabolism of histidine is absent in *T. brucei* and a functional urea cycle is missing across the trypanosomes.

In spite of the extrapolation possible from genome analysis, little is known about amino acid usage and consumption in BSF trypanosomes. Cysteine is an essential amino acid for *in vitro* cultures (Duszenko *et al.*, 1992). The enzyme L-threonine 3-dehydrogenase is important in metabolising threonine. L-threonine metabolism takes place in the mitochondrion producing glycine and acetyl CoA. BSF trypanosomes, however, lack a complete functional mitochondrion, and nothing is known so far about the subcellular localization of the threonine metabolism in this life form. Recently, Nano LC-MS/MS proteomic analysis has revealed the presence of an L-threonine 3-dehydrogenase (Tb927.6.2790) enzyme in the glycosome of BSF trypanosomes (Colasante *et al.*, 2006), suggesting that BSF trypanosomes do metabolise threonine. However, the authors were unable to postulate a reason why this enzyme is localised in the glycosome of the BSF trypanosome.

One important use of amino acids is in the regulation of cell osmolarity. Osmoregulation is the active regulation of the osmotic pressure of fluids to maintain the homeostasis of water content, which, in trypanosomes is closely linked to amino acid accumulation and release. The mechanics of osmoregulation has been more closely studied in *Leishmania*. Accumulated amino acids provide the cell with an osmotic reservoir, which is readily utilised for osmoregulation. When *Leishmania* promastigotes are exposed to a sudden decrease in osmolality, they initially swell but subsequently undergo shrinkage to compensate (Blum *et al.*, 1999). This regulatory volume decrease (RVD) response

mechanism involves anionic amino acid channels (HAAC – hypotonic-activated amino acid anion channel), which allow neutral and acidic amino acids to be released from the cells. The HAAC system has been likened to volume activated anion channels at work in mammalian cells that mediate the efflux of small organic osmolytes.

In a hypotonic environment, osmosis causes water to flow into the cell, leading to swollen cell morphology. The release of amino acids by *Leishmania* promastigotes has been shown to be proportional to the osmotic stress placed on the cell (Blum *et al.*, 1999). That is to say, that the proportionality was independent of ionic strength and proportional to the imposed osmotic gradient. Alanine, proline and glutamate (among others) are the principal amino acids implicated in osmoregulation in *Leishmania*.

Other cell species use the dissipation of potassium and chloride ions (linked to water release) for correcting volume changes (Vieira *et al.*, 1996). The use of osmotic reservoirs of organic solutes means adaptive regulation of volume changes. *Leishmania* promastigotes swell in hypotonic media but recover their resting shape and volume within 15–20 mins. In *T. brucei* swelling of cells by decreasing the osmotic strength of the extracellular medium has been used for membrane permeability experiments and as a method for incorporating non-penetrating (non-transported) low molecular weight molecules into cells (Voorheis *et al.*, 1980).

3.1.2 Genes of the Amino Acid Transporter Family

Large amino acid transporter gene families have been identified from the tritryp genome sequences (Berriman *et al.*, 2005; Jackson, 2007). Across the 11 megabase chromosome of the African trypanosome, 6 chromosomes contain repetoires of amino acid transporters. Chromosome 8 of the *T. brucei* genome is rich in amino acid transporter genes and two transporter islands were located – one containing Tb927.8.4700 (AATP6, AAT5) and the other containing Tb927.8.7610 (AATP1, AAT7), Tb927.8.7600 (AATP2, AAT7) and Tb927.8.7680 (AATP4, AAT7) in a tandem array (Figure 3-1). AATP6 and AATP1 were chosen for knock-down functionality investigations. At the same time, a candidate amino acid transporter that showed the most sequence divergence within the family was chosen (Figure 3-2).

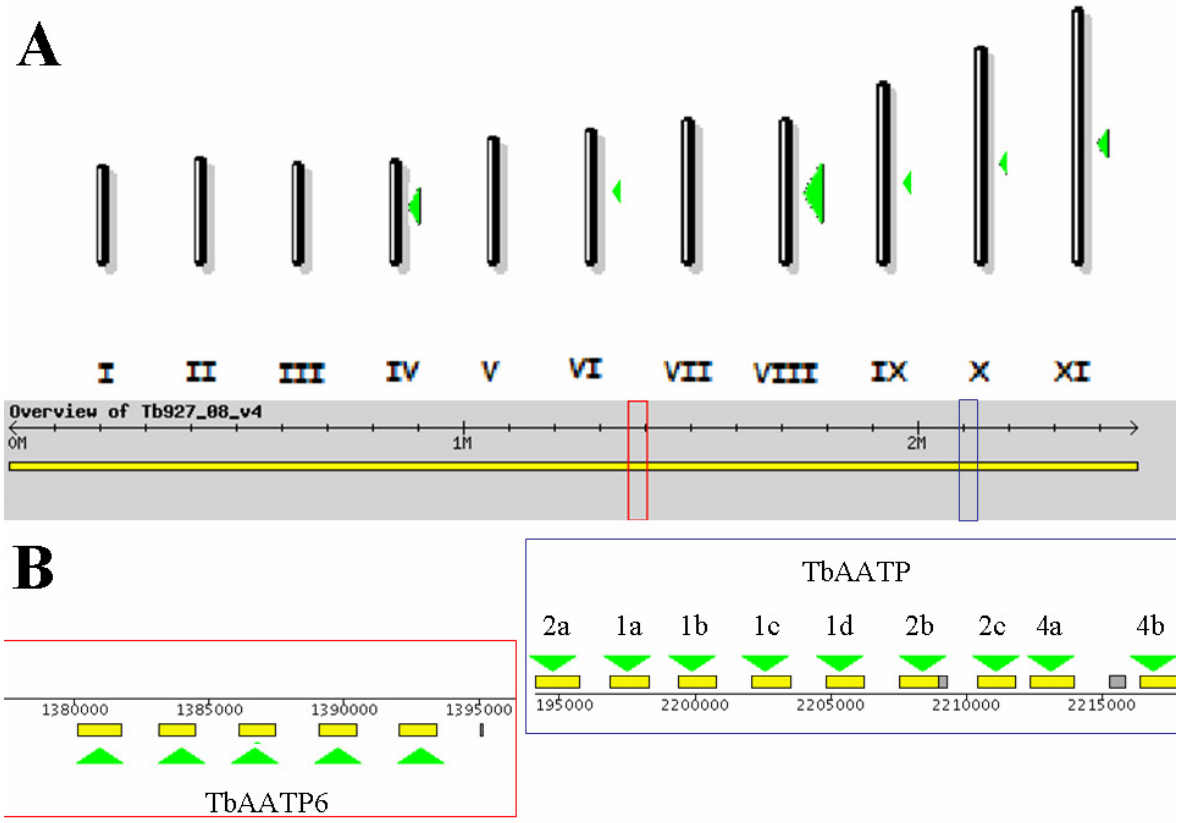


Figure 3-1: Amino Acid Transporter location (TbAATP6 and TbAATP1).
All 11 megabase chromosomes of the *T. brucei* genome, showing relative amino acid transporter densities (A). Chromosomal location of two identified amino acid transporter islands along chromosome 8. Gene arrays of individual transporter islands (B), showing TbAATP6 five gene array (red box) and the downstream array containing TbAATP1 (AAT7), TbAATP2 (AAT7) and TbAATP4 (AAT7).

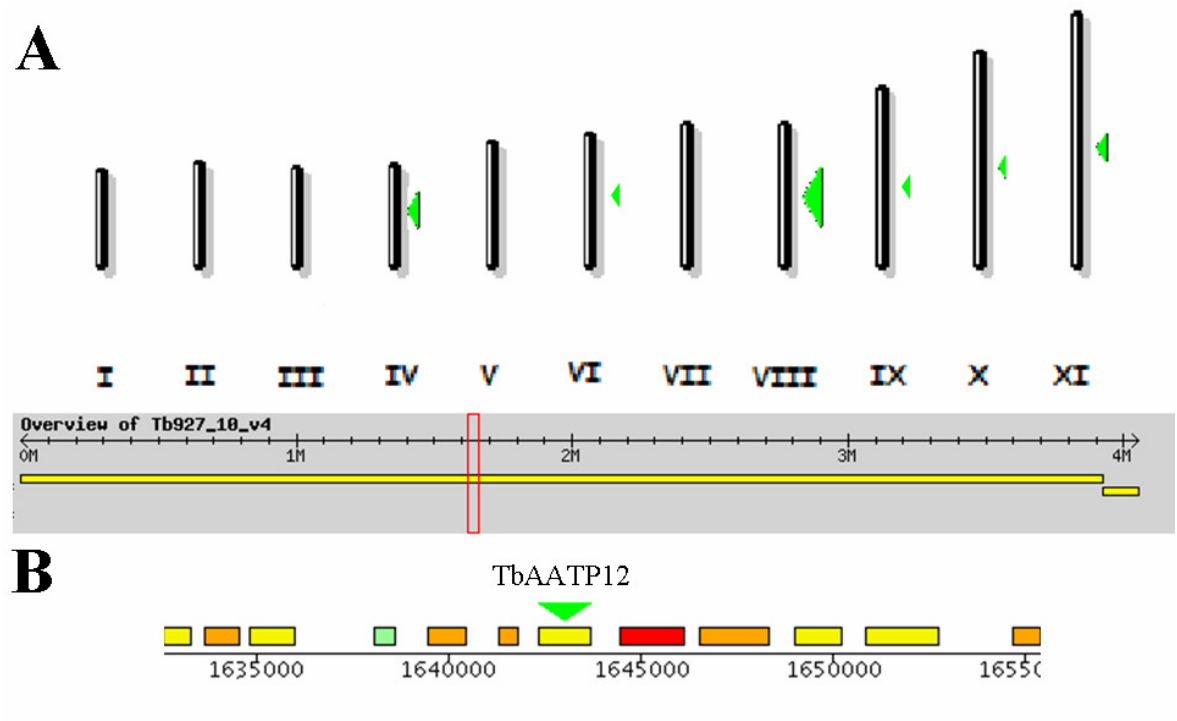


Figure 3-2: Amino Acid Transporter location (TbAATP12).
All 11 megabase chromosomes of the *T. brucei* genome, showing relative amino acid transporter densities (A). Chromosomal location of TbAATP12 (AAT13) along chromosome 10 (B).

Of the three gene groups selected AATP1 has no homeologs in *Leishmania* or *T. cruzi* genomes, whereas AATP12 is present in all three species and an AATP6 homolog is only found in *T. cruzi* (Jackson, 2007). In general, TbAATP1 shows a ≥95% similarity within the group, TbAATP2 shows a ≥85% similarity within the group, TbAATP4 genes are 99% similar to one another, and TbAATP6 genes show a ≥98% similarity within the group. TbAATP1 is most closely related to TbAATP2 (>70%) and least to TbAATP6 (~40%). It was hoped that these investigations would provide information into the function and regulation of closely related, co-located transporters. Genetic modification of trypanosomes (removal of transporter genes via gene knock out or knock down of transcripts by RNAi-based systems) should help to clarify the role of individual transporters to net measured uptake of amino acids in these cells. As well as evaluating the function of *T. brucei* AATP genes.

Previously, Dr. I. Accoceberry amplified segments of the *T. brucei* TbAATP1 gene, from *T. brucei* strain 427 genomic DNA using the following primers:

5'-ATATGGATCCAACATCGGATCAACTACCATTG-3', sense from position 201 to 223 of the TbAATP1 coding region, including a *BamHI* site (underlined).

5'-ACATAAGCTTACCAAAGAAGGCTGTCAGTGCA-3'antisense complementary to positions from 915 to 936, including a *HindIII* site (underlined), for amplification of 735 bp (N terminal region).

The construct carrying a 606 bp fragment of TbAATP1 gene was cloned into the p2T7Ti vector (LaCount *et al.*, 2000) for use in RNAi experiments. Two cloned lines of PCF trypanosomes generated for RNAi-based experiments were selected by Dr. I Accoceberry – the lines were named IC8 and 4E8. Preliminary experiments indicated that the TbAATP1 transporter is a carrier of small, uncharged amino acids with specificity for L-serine, L-threonine, L-alanine, glycine, L-cysteine and L-asparagine. Here, I set out to confirm L-threonine uptake via TbAATP1 as well as search for a functional role of this newly characterised amino acid transporter.

TbAATP6 is one of the three groups of amino acid transporter genes upregulated in procyclic forms (Tb927.4.4730, Tb927.8.4700, and Tb927.4.3990) (Brems *et al.*, 2004). In all, RNAs from BSF and PCF trypanosomes were used as templates for the generation of fluorescent cDNA probes. The resulting probes were hybridised to the generated genomic arrays, revealing differentially regulated genes. AATP6 was chosen for knock-down functionality investigations.

TbAATP12 was chosen under the basis of it being an 'orphan' (one that bears the least similarity to any of its neighbours). This 'orphan' amino acid transporter (Figure 3-2), located on chromosome 10, was termed TbAATP12. TbAATP12 shows only a 15% homology to the other amino acid transporter along the chromosome (Tb10.6k15.0450, AAT14) and a 36% homology to its closest related amino acid transporter (Tb927.4.4860, AAT4).

Genetic modification of trypanosomes (removal of genes via knock-out or knock-down of transcripts) can help identify the roles of amino acid transporter genes in trypanosomes. I report here the use of RNAi to evaluate the function of candidate amino acid transporter genes from *T. brucei*.

3.2 Results

3.2.1 Identification of Genes

The GeneDB *Trypanosoma brucei* database was screened using the name "amino acid transporter." From the database 46 genes with the molecular function, biological process and cellular component corresponding to amino acid transport were revealed (Table 2). The GeneDB annotations of amino acid transporter gene products are based on inferred electronic annotation (annotations based on "hits" in sequence similarity searches), and inferred from sequence or structural similarity to known amino acid transporters.

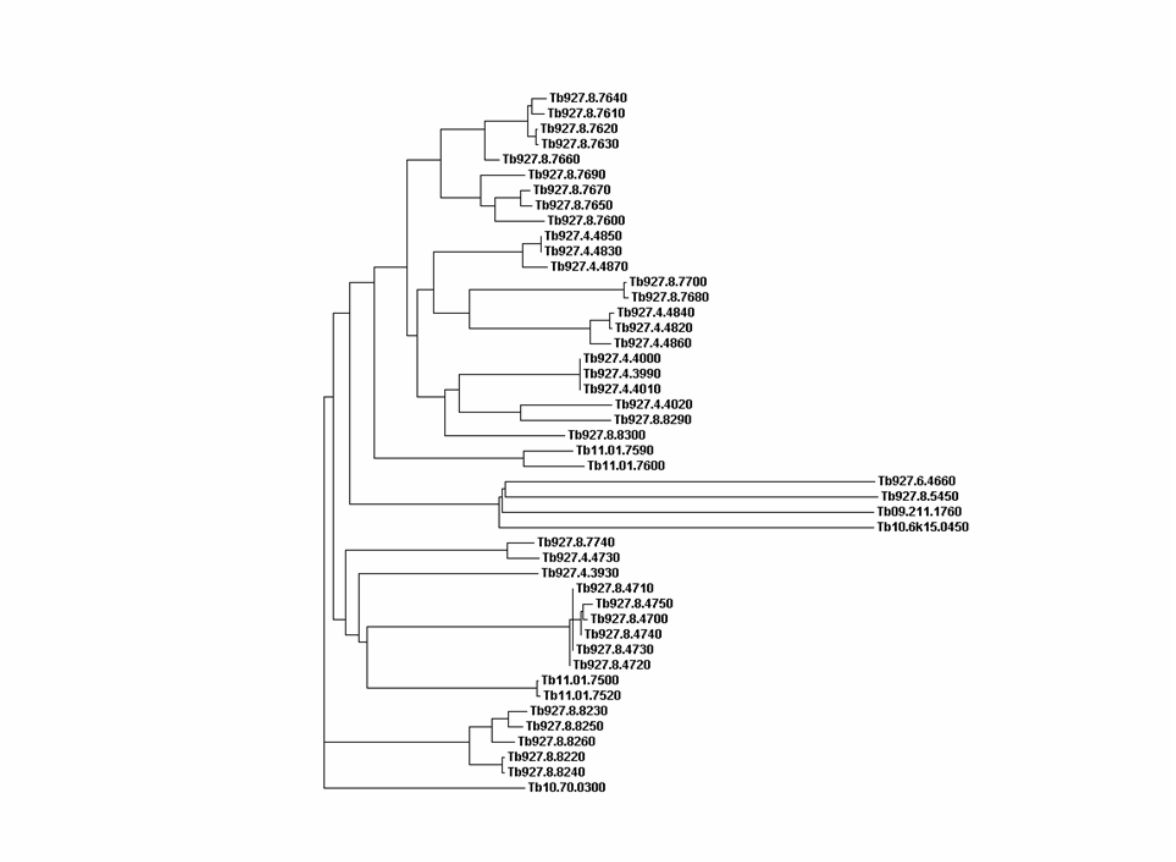


Figure 3-3: Dendogram of aligned AATP genes.
DNA sequence alignment of all 46 putative amino acid transporter genes as annotated by the *T. brucei* genome database.

All 46 genes were aligned using Clustal W and grouped based on homology, as a guide to the level of relationship exhibited by all genes, with a view to later functional expression experiments to find a phenotype. From the alignments, close clustering of gene groups

emerged (Figure 3-3). Closely related genes, showing a sequence identity greater than 75% were classed into AATP groups and given new AATP numbers (Table 2).

Parallel Classification			
Gene I.D.	AATP# (from GeneDB)	New AATP# (by alignments)	AAT Locus
Tb927.8.7610	AATP1		AAT7
Tb927.8.7620	AATP1		AAT7
Tb927.8.7630	AATP1		AAT7
Tb927.8.7640	AATP1		AAT7
Tb927.8.7660	AATP1		AAT7
Tb927.8.7600		AATP2	AAT7
Tb927.8.7650		AATP2	AAT7
Tb927.8.7670		AATP2	AAT7
Tb927.8.7690		AATP2	AAT7
Tb927.8.7680		AATP4	AAT7
Tb927.8.7700		AATP4	AAT7
Tb927.4.4830		AATP3	AAT4
Tb927.4.4850		AATP3	AAT4
Tb927.4.4870		AATP3	AAT4
Tb927.4.4840	AATP7		AAT4
Tb927.4.4820	AATP10		AAT4
Tb927.4.4860	AATP8		AAT4
Tb927.4.3990		AATP13	AAT2
Tb927.4.4000		AATP13	AAT2
Tb927.4.4010		AATP13	AAT2
Tb927.4.4020		AATP5	AAT2
Tb927.8.8290	AATP5	AATP5	AAT10
Tb927.8.8300		AATP9	AAT10
Tb11.017590		AATP14	AAT17
Tb11.01.7600		AATP14	AAT17
Tb927.6.4660	CAATP	ATP15	-
Tb927.8.5450		AATP16	AAT6
Tb09.211.1760		AATP17	AAT11
Tb10.6k15.0450		AATP18	AAT14
Tb927.8.7740		AATP11	AAT8
Tb927.4.4730	AATP11	AATP11	AAT3
Tb927.4.3930		AATP20	AAT1
Tb927.8.4700	AATP6	AATP6	AAT5
Tb927.8.4710		AATP6	AAT5
Tb927.8.4720		AATP6	AAT5
Tb927.8.4730		AATP6	AAT5
Tb927.8.4740		AATP6	AAT5
Tb927.8.4750		AATP6	AAT5
Tb11.01.7500		AATP21	AAT16
Tb11.01.7520		AATP21	AAT16
Tb927.8.8220		AATP22	AAT9
Tb927.8.8230		AATP22	AAT9
Tb927.8.8240		AATP22	AAT9
Tb927.8.8250		AATP22	AAT9
Tb927.8.8260		AATP22	AAT9
Tb10.70.1170			AAT12
Tb11.02.4520			AAT15
Tb10.70.0300		AATP12	AAT13

Table 2: Amino acid transporter genes from *T. brucei*.

In general, closely located genes (along the same chromosome) show a high similarity to each other. For example, AATP1 (Tb927.8.7610/20/30/40/60) and AATP2 (Tb927.8.7650/70/90) show a ≥70% identity to each other. The identity found among tandem copies (see later) indicates that duplication of these genes constitutes a recent dynamic event. It is possible that gene duplication resulted in close forms of co-located transporters and over time the slight divergence in sequence emerged.

The advantage of indexing amino acid transporter genes by sequence homology rather than genomic position (Jackson, 2007) means individual genes with related function can be grouped, leading to easier and more efficient downstream functional genomic strategies. With the amount of genetic redundancy in amino acid transporter genes, it is hard to assess what level of homology is required between genes that would translate to function.

3.2.2 TbAATP1 & Growth of PCF trypanosomes

TbAATP1 RNAi cell lines were used to explore a role for this transporter system in growth. Cells of the 4E8 clone (previously derived by Dr Accoceberry) were grown in the presence and absence of tetracycline. Furthermore, loss of threonine was explored in differing medium conditions. When D-glucose is not the main carbon source PCF trypanosomes have more reliance on proline for growth (Lamour *et al.*, 2005). It is possible that other amino acids become more essential for growth in the absence of glucose.

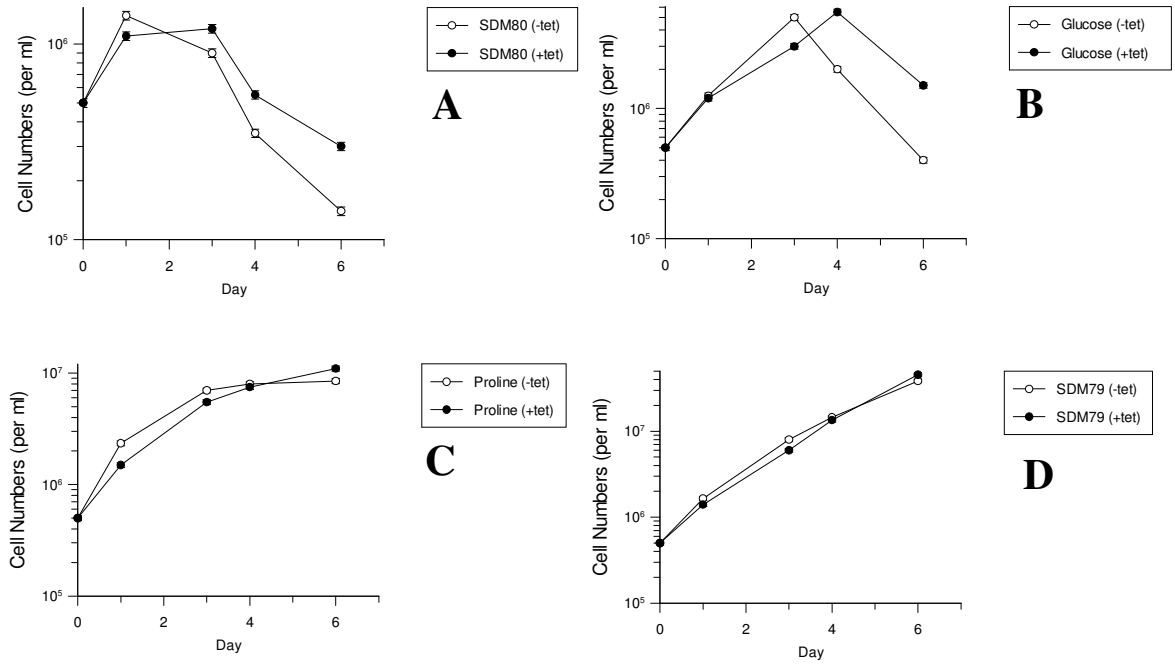


Figure 3-4: Growth of TbAATP1 4E8 RNAi cell lines.
Tetracycline induced an uninduced, grown in SDM80 (panel A), SDM80 plus 10 mM D-glucose (panel B), SDM80 plus 10 mM L-proline (panel C), and SDM79 (panel D).

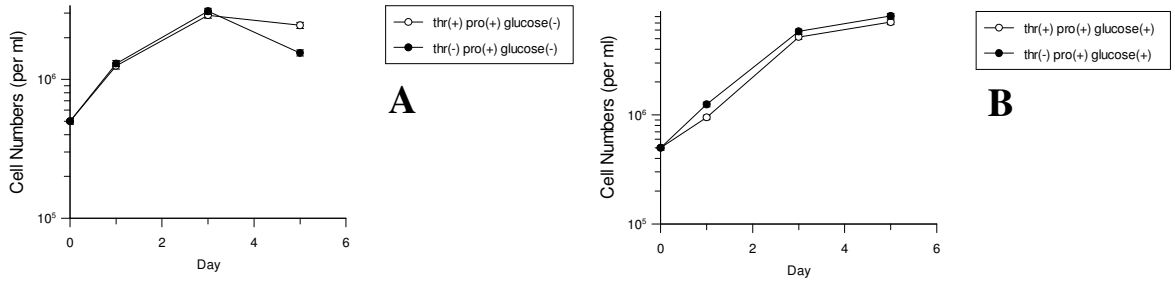


Figure 3-5: Growth of PCF trypanosomes (strain 427) in threonine deficient medium.
SDM80 deficient of L-threonine supplemented with 10 mM threonine and 10 mM proline and 10 mM glucose.

It seems apparent here that, for the ΔTbAATP1-i cell line, there is no significant difference in growth when cells are utilising differing carbon sources. However, with RNAi knock-down, total protein loss of function is seldom attained. With this in mind I wanted to test threonine's contribution to cell growth by using a more robust control. Wild type PCF trypanosomes were grown in threonine deficient medium to ascertain if PCF trypanosomes were capable of growing without the amino acid. It was found that there is no growth defect seen when growing wild type PCF trypanosomes in L-threonine deficient medium with L-proline or D-glucose as the primary carbon source. Cells grown in L-threonine

deficient medium showed no growth disadvantage over controls, even in the absence of glucose. Growth during mid-log phase of RNAi cell lines was explored by systematic passaging of cells.

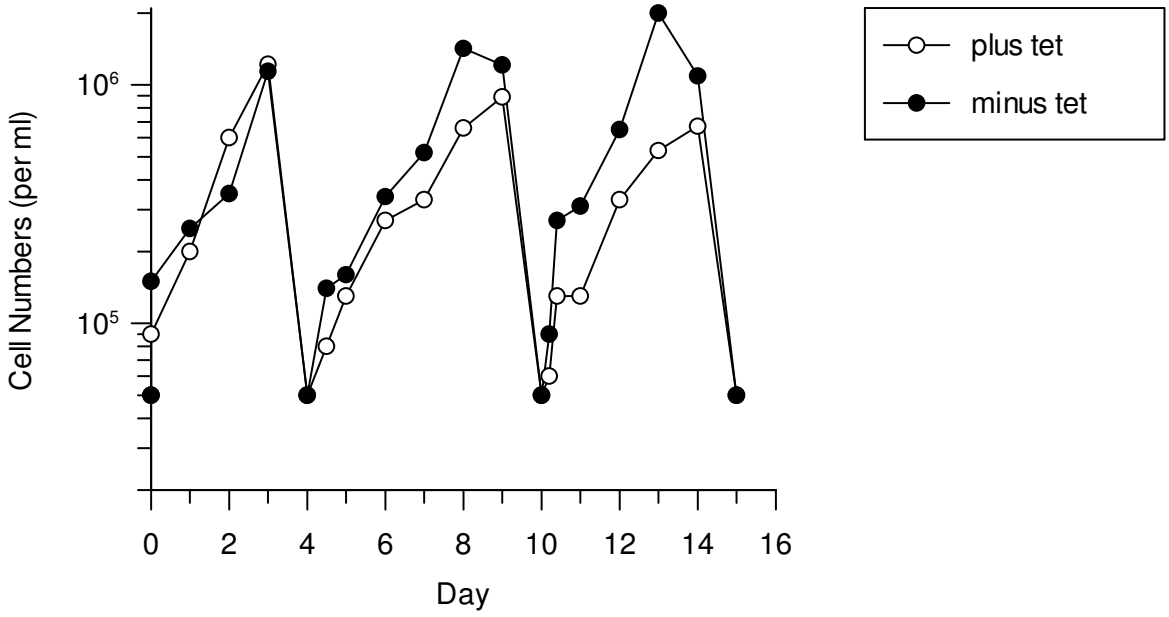


Figure 3-6: Growth counts of TbAATP1 4E8 RNAi cell lines.
Induced and uninduced cell lines (SDM79).

Induced cells showed stunted growth over time (after the first passage). In comparison with non-induced cell lines, they fail to reach comparable numbers. With RNAi non-responsiveness to tetracycline can occur after prolonged tetracycline exposure (La Count, personal communication). With the growth defect seen (Figure 3-6) it was important to determine TbAATP1 transporter activity through measuring threonine uptake.

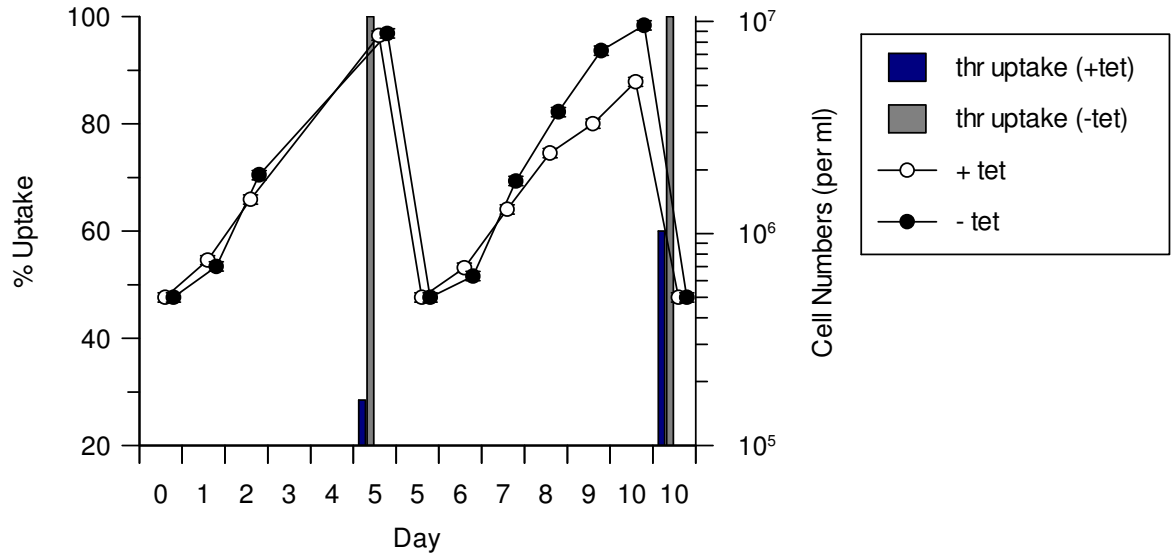


Figure 3-7: Growth and measure of threonine uptake.
Tetracycline induced and uninduced TbAATP1 4E8 RNAi cell lines (this result represents data from 3 similar repeats).

By day 5, threonine uptake shows a ~70% reduction and remained reduced by day 10, however, only at ~40%. It is possible that it is the initial loss of transporter activity that results in the stunted growth. Although, a role for threonine in growth has been ruled out, a role for TbAATP1 in growth has not been ruled out. It is possible that the cumulative reduction of six amino acids (threonine, cysteine, glycine, serine, asparagine, and alanine) is what leads to the growth defect seen, but the effect is time dependent. Trypanosomes contain high intracellular pools of amino acids which they utilise for cellular processes (such as osmoregulation). It is possible that with TbAATP1 not-functioning, the cells are able to use their intracellular pools to compensate. However, once the pool is exhausted, defective growth is seen.

L-threonine's role in acetate formation and downstream lipid metabolism is a potential avenue that needs further investigation. L-threonine has been proposed as a major supply of acetyl-CoA for fatty acid biosynthesis (Cross *et al.*, 1975). Investigations into the changes in the lipid profile of knock-down cell lines are ongoing (Dr Terry Smith, University of Dundee). By diminishing threonine uptake the cells might end up altering their lipid profiles by shifting dependency on synthesised versus acquired fatty acids.

3.2.3 L-threonine Transport in T. brucei

Time dependence of L-threonine uptake was studied in tetracycline induced and uninduced ΔTbAATP1-i cells (96 hour induction); by incubating cells for different time periods in CBSS buffer (Appendix 8.3) containing 10 μM [^{3}H] L-threonine.

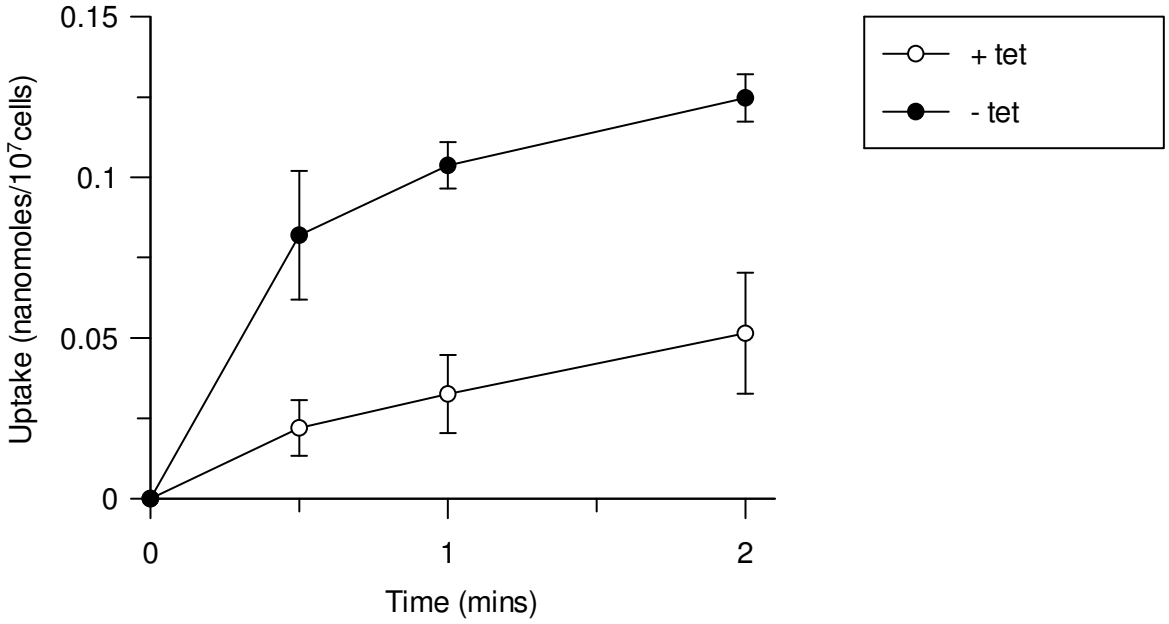


Figure 3-8: Threonine uptake.
Uptake of 10 μM L-[^{3}H] Threonine in TbAATP1 4E8 RNAi cell lines (plus and minus tet induction) over a 2 minute time period (n=3, Error bars = ±SEM).

Measured internalised threonine is significantly reduced in induced ΔTbAATP1-i cell lines, showing a 60-70% reduction in uptake over the 2 min incubation period. Reduction of threonine uptake is never total probably due to the fact that RNAi knock-down is never total. It was previously shown that uptake of radiolabeled serine was inhibited by excess of unlabelled serine, L-threonine, L-alanine, glycine, L-cysteine, and L-asparagine (Accoceberry, unpublished). Therefore, TbAATP1 substrate specificity was further examined by measuring uptake of radiolabelled L-threonine (at 10 μM) in the presence of 10 mM of each of the 20 non-radiolabelled amino acids (Figure 3-9).

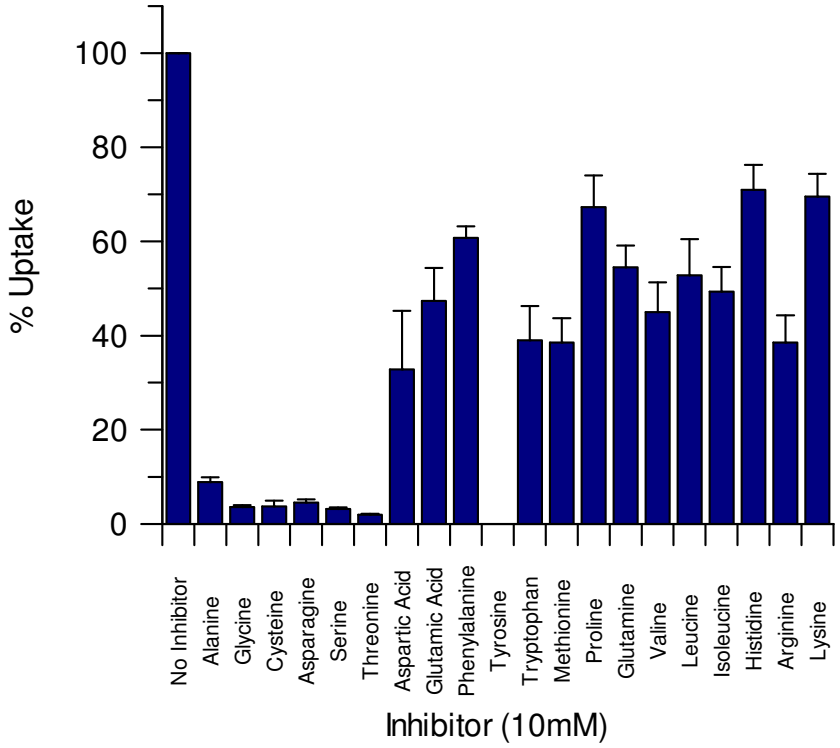


Figure 3-9: Threonine inhibition.
Inhibition of L-[^{3}H] Threonine uptake. Uptake in PCF trypanosomes of 10 µM threonine in the presence of all 20 amino acids at a concentration of 10 mM. Uptake shown as a percentage of control with no inhibitor (L-Tyrosine is not soluble at a concentration of 10 mM under the conditions required for this experiment. Consequently inhibition of uptake was not determined in the presence of tyrosine) (n=4, Error bars = ±SEM).

Results represent the percentage of L-threonine uptake over a time period of 30 sec against a control containing only L-[^{3}H] threonine. All amino acids show some degree of inhibition but maximal inhibition of uptake of radiolabelled L-threonine is seen in the presence of cold L-serine, L-threonine, glycine, L-cysteine, L-alanine and L-asparagine. Biochemically, this suggests a common route of entry for these six amino acids.

I wanted to ascertain the order of preference of substrate for TbAATP1. I measured uptake of radiolabeled serine in the presence of unlabelled serine and threonine (up to 1 mM) to find out which inhibited to a greater degree.

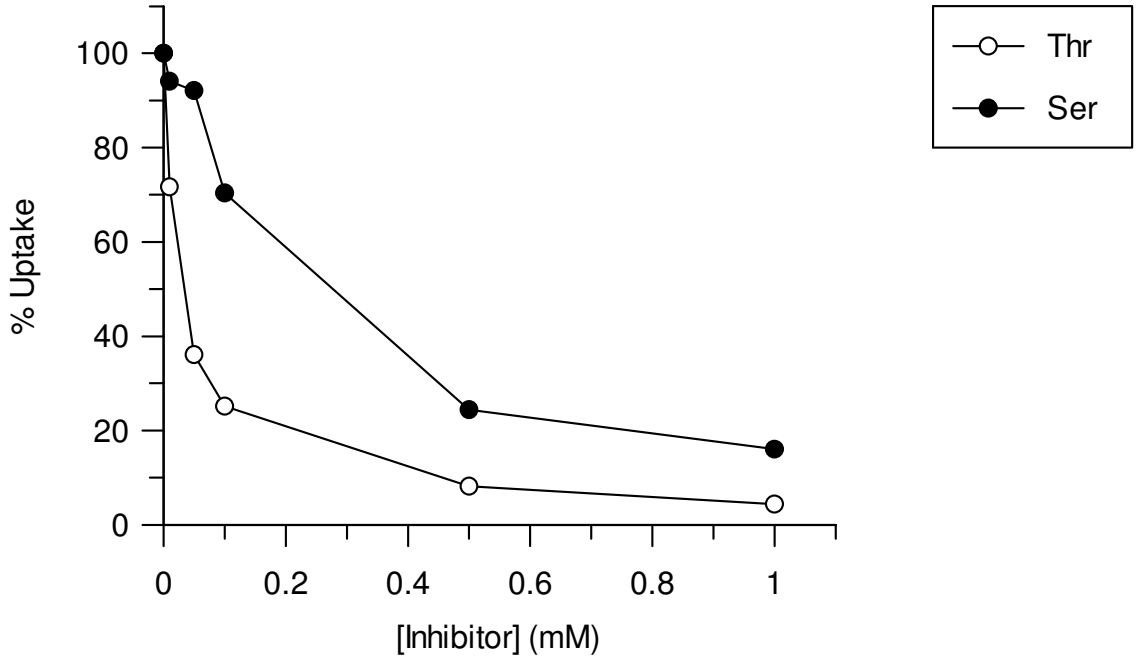


Figure 3-10: Serine inhibition.
Uptake of 10 µM L-[^{3}H] Serine in the presence of increasing concentrations of cold serine and threonine (no inhibitor, 10 µM, 50 µM, 100 µM, 500 µM, and 1 mM) (n=1).

Inhibition gave IC_{50} values in the micro molar range, with serine giving an IC_{50} value of 147.1 µM ± 82.3 and threonine giving an IC_{50} value of 26.7 µM ± 1.5. Inhibition of radiolabeled serine is more complete and to a greater degree in the presence of threonine. This suggests that TbAATP1 has a higher affinity for threonine than it does for serine.

In order to ascertain affinities for each substrate that uses the threonine-specific transporter system a more detailed inhibition assay was performed. L-threonine was used as the primary radiolabel due to the fact that it was the most specific for this transporter system (uptake of cold L-threonine inhibited radiolabelled L-serine by a greater degree at the same concentration). Uptake of radiolabelled L-threonine (10 µM), in the presence of 0-10 mM of each of the inhibiting amino acids, was studied using a single 30 sec time point.

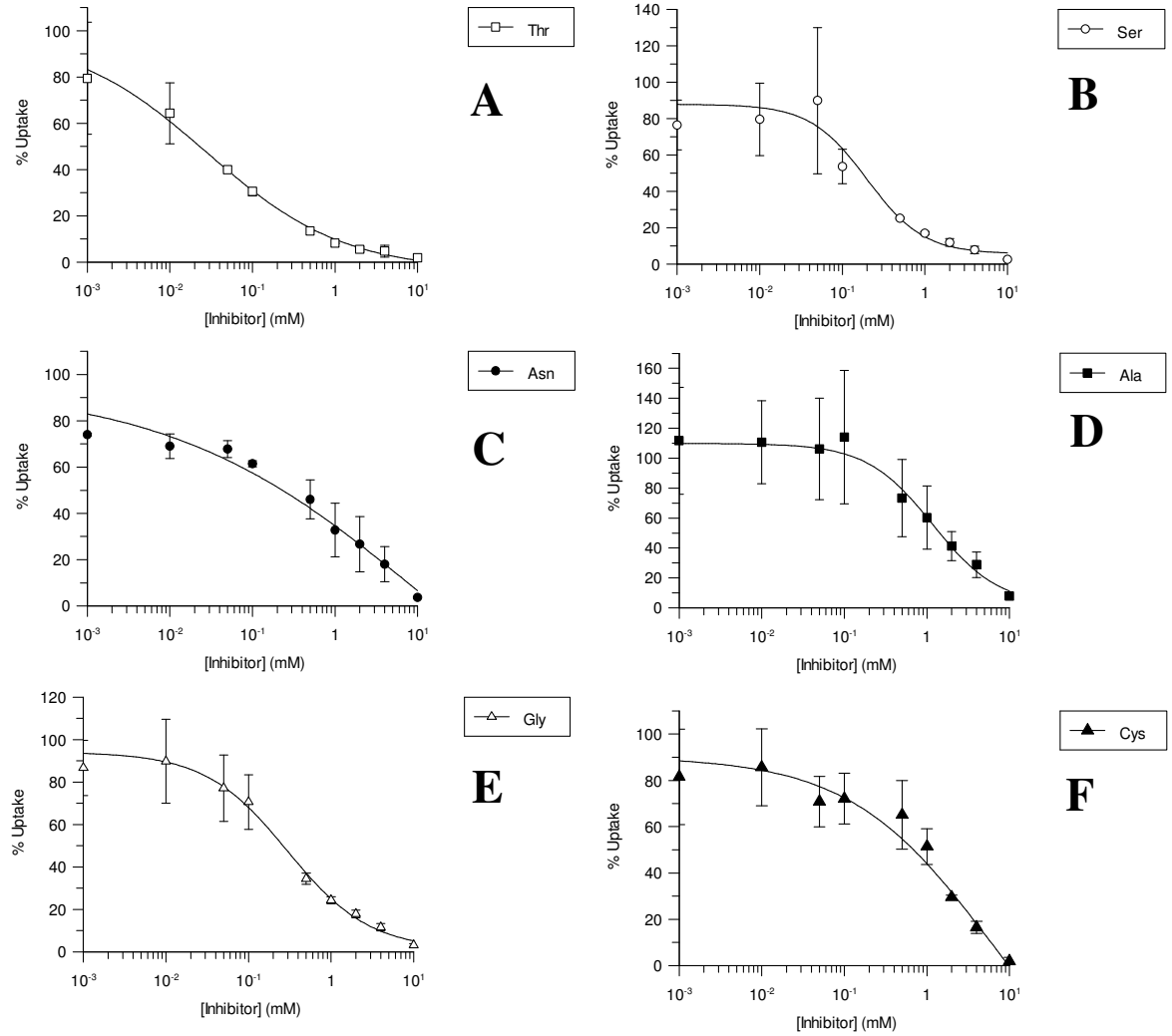


Figure 3-11: Threonine inhibition by AATP1 specific amino acids.
Inhibition of 10 µM L-[3H] Threonine using cold threonine (A), serine (B), asparagine (C), alanine (D), glycine (E), and cysteine (F) over a range of concentrations (no inhibitor, 1 µM, 10 µM, 50 µM, 100 µM, 500 µM, 1 mM, 2 mM and 4 mM). Uptake shown as a percentage of control with no inhibitor (n=3, Error bars = ±SEM).

Using a range of concentrations can distinguish between 'weak' and 'partial' inhibition. In each case these amino acids gave a progressive dose-dependent inhibition. Suggesting the order of affinity for AATP1 being L-threonine>>L-serine>>Glycine>>L-alanine>>L-cysteine>>L-asparagine.

	IC_{50}	SEM
Threonine	27.9 µM	5.3
Serine	197.3 µM	91.9
Glycine	296.2 µM	71.4
Cysteine	5.74 mM	4.91
Asparagine	9.57 mM	5.35
Alanine	1.17 mM	0.335

Table 3: Calculated IC_{50} for the TbAATP1 specific amino acids.

L-threonine, L-serine and glycine have the highest affinities for TbAATP1, showing IC_{50} values in the micro molar range. The calculated IC_{50} values refer to the amount of inhibitor required to inhibit uptake of a particular amino acid by 50%.

Kinetic parameters for the mode of threonine uptake were determined. Measuring initial velocity of L-threonine transport against substrate concentration (starting at 200 µM threonine) for the TbAATP1 cell line grown with and without tetracycline revealed a typically hyperbolic curve, and V_{max} and K_m values were calculated by non-linear regression.

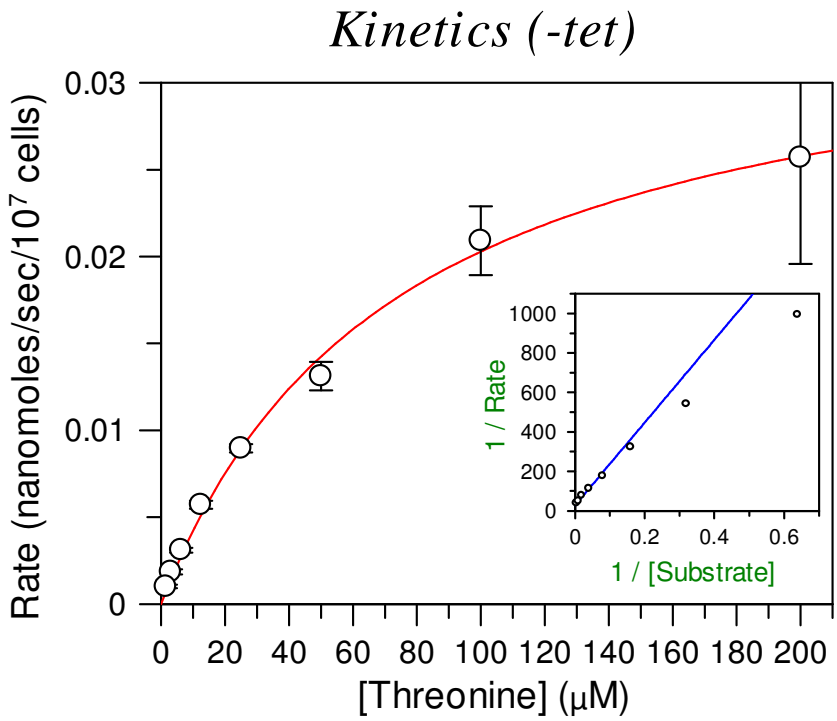


Figure 3-12: Kinetics of threonine uptake in uninduced TbAATP1 RNAi cell lines.
Uptake of L-[^{3}H] Threonine over a range of concentrations at a single 30 sec time point (n=3).

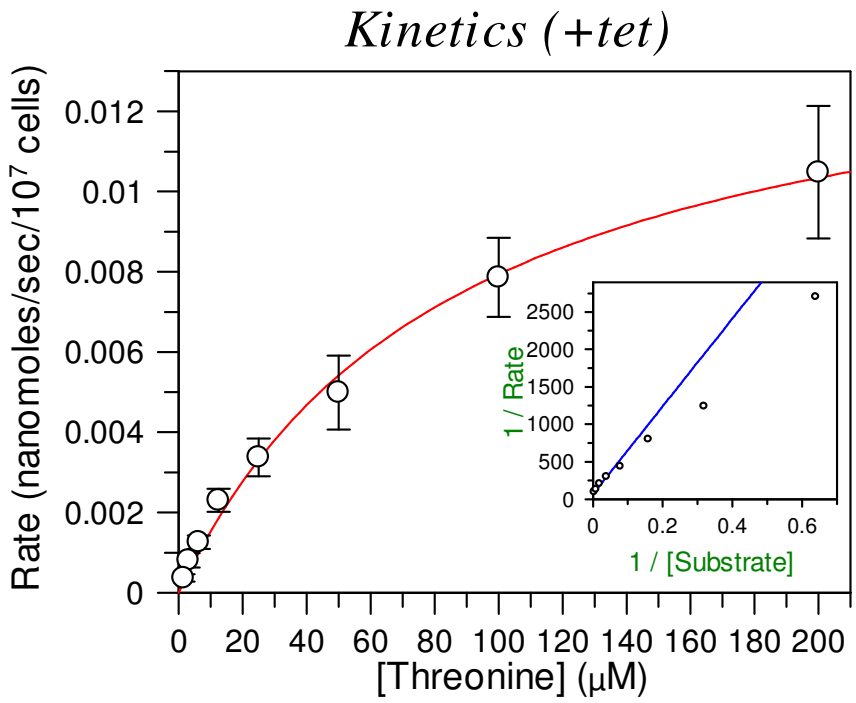


Figure 3-13: Kinetics of threonine uptake in tetracycline induced TbAATP1 RNAi cell lines. Uptake of L-[^{3}H] Threonine over a range of concentrations at a single 30 sec time point (n=3).

The apparent kinetic behaviour of L-threonine uptake is comparable to the inhibition constant (IC_{50}) previously found. Inhibition of uptake of radiolabelled L-threonine by cold L-threonine over a range of concentrations gave an inhibition constant of 27.96 µM. Here, kinetic constants were calculated by non-linear regression using the Michelis-Menten equation. For the uninduced cell line a K_m value of 73.925 µM and a V_{max} of 35.3 picomols/sec/10^7 cells were calculated, whereas for the induced cell line a K_m value of 86.967 µM and a V_{max} of 14.8 picomols/sec/10^7 cells were calculated.

3.2.4 Osmoregulation

When *L. donovani* are challenged with an acute decrease in osmolarity, the parasites rapidly release amino acids of which Ala, the major osmolyte in cells, accounted for half of the total amino acid loss (Darling & Blum, 1990). Osmoregulation in PCF trypanosomes was tested here in the context of the RNAi cell line TbAATP1. TbAATP1 is a transporter of small, uncharged neutral amino acids (alanine, glycine, cysteine, serine, threonine and asparagine). The proposition is that with transporter availability cells will be unable to regulate cell volume and shape under hypotonic stress. All observations for this cell line were done at 72 hrs post tetracycline induction.

Three different hypotonic conditions was used, simply differing in water content (0, 25% and 50% water). Cells were monitored by microscopy after a 30 min time period to judge the level of swelling that initially occurred and the change in cell shape.

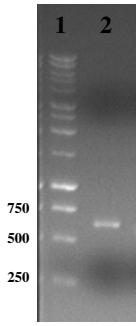
Overall, there was no difference in the amount of general swelling when comparing induced and uninduced cell lines. It is entirely possible that the length of time needed for TbAATP1 induced cells to recover resting shape within hypotonic media is altered. Hence, no difference in swell shape is seen between the two cell lines. This could be due to the fact that although there is a common transport route for these amino acids, other general amino acid transport mechanisms could contribute. As well as the fact that release of alanine to compensate for swelling could easily be substituted with another amino acid pool. Although, the TbAATP1 transporter has here not been shown to be implicated in osmoregulation, other systems could be at play.

3.2.5 Cloning of TbAATP6 & TbAATP12 RNAi Constructs

In this study I tested the ability of the p2T7Ti vector to generate, by RNAi knockdown, mutant phenotypes for TbAATP12 and the TbAATP6 set of genes not previously characterised in *T. brucei*. I attempted to inhibit expression of the *T. brucei* TbAATP6 and TbAATP12 genes in PCF trypanosomes. A previously developed *T. brucei* cell line 29-13, engineered to express T7 RNA polymerase and the tetracycline repressor, was used. A 600 bp fragment from the TbAATP6 open reading frame was inserted between the two opposing T7 promoters of the p2T7Ti vector that are both regulated by tetracycline repressors. A 554 bp fragment and a 620 bp fragment from the TbAATP12 open reading frame was inserted between the two opposing T7 promoters of the p2T7Ti vector that are both regulated by tetracycline repressors.

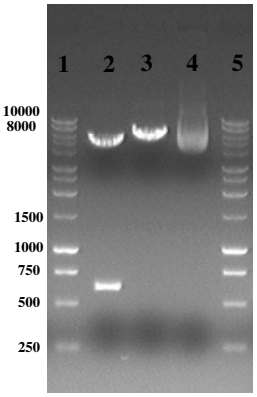
A 3'end segment of the *T. brucei* "TbAATP6" gene was amplified from *T. brucei* strain 427 genomic DNA using the following primers:

5'- ATATGGATCCATTGTCAATCTTCATGTTTTC-3', sense from position 980 to 1001 of the TbAATP6 coding region, including a *BamHI* site (underlined), and 5'-ACATCTCGAGATTGAGGCAGCCGTACCAAAC-3', antisense complementary to positions from 1559 to 1580, including a *XhoI* site (underlined), for amplification of 600 bp (C terminal region) (Figure 3-14).



Purified PCR product
Lane 1: 1 kb Ladder Marker
Lane 2: TbAATP6 purified PCR product

Figure 3-14: Ethidium bromide stained gel showing PCR amplification of a segment of TbAATP6.



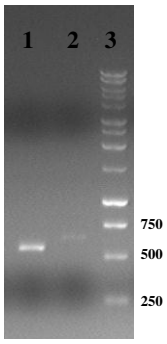
p2T7Ti/TbAATP6
Lane 1: 1 kb ladder marker
Lane 2: BamHI/XhoI digest
Lane 3: NotI linearisation
Lane 4: uncut
Lane 5: 1 kb ladder marker

Figure 3-15: Ethidium bromide stained gel showing excision of p2T7Ti ligated TbAATP6 insert.

5'end and 3'end segments of the *T. brucei* "TbAATP12" gene were amplified from T. brucei strain 427 genomic DNA using the following primers:

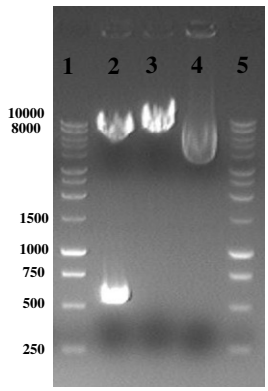
5'- ATATGGATCCATGGTTGTGAATTCTGATGGG-3', sense from position 1 to 21 of the TbAATP12 coding region, including a *BamHI* site (underlined), and 5'-ACATCTCGAGAAAAATGTTGTTAGGCGGACA-3', antisense complementary to positions from 533 to 554, including a *XhoI* site (underlined), for amplification of 554 bp (N terminal region) (Figure 3-17).

5'- ATATGGATCCGGGATTTGGTATTTTTATCTT-3', sense from position 740 to 761 of the TbAATP12 coding region, including a *BamHI* site (underlined), and 5'-ACATCTCGAGATTGATAATCGCATAAATCGT-3', antisense complementary to positions from 1518 to 1539, including a *XhoI* site (underlined), for amplification of 620 bp (C terminal region) (Figure 3-16).



Purified PCR product
Lane 1: TbAATP12a PCR product
Lane 2: TbAATP12b PCR product
Lane 3: 1 kb Ladder Marker

Figure 3-16: Ethidium bromide stained gel showing both fragments of TbAATP12 amplified by PCR.



p2T7Ti/TbAATP12a
Lane 1: 1 kb ladder marker
Lane 2: BamHI/XhoI digest
Lane 3: NotI linearisation
Lane 4: uncut
Lane 5: 1 kb ladder marker

Figure 3-17: Ethidium bromide stained gel showing excision of p2T7Ti ligated TbAATP12a insert.

Transfection of the PCF cell line 29-13 with the RNAi constructs directed against TbAATP6 and TbAATP12 proved moderately efficient. The cells survived 14 day phleomycin selection (0.1 µg/ml) and were subsequently cloned out by limiting dilution.

However, for TbAATP6, viable cells grew very slowly initially even in the presence of up to 75% conditioned medium.

3.2.6 Northern Blot Analysis

The effectiveness of RNAi on mRNA levels and the correlation between the growth defects and mRNA levels was examined. RNA was extracted from clonal lines before and after induction with tetracycline. Total isolated RNA was then subjected to Northern analysis with a probe derived from the coding region of TbAATP6 and TbAATP12.

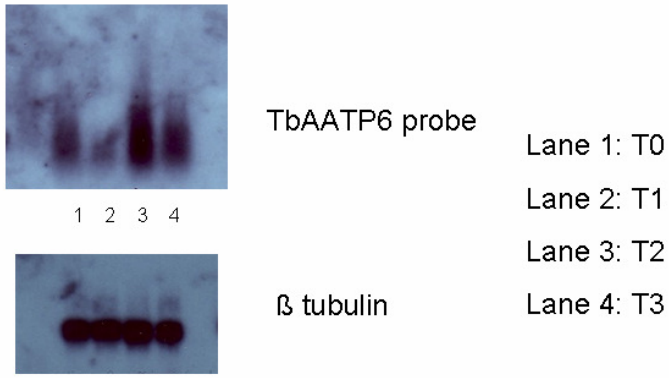


Figure 3-18: Northern blot analysis of RNA from TbAATP6 RNAi cell lines.
Northern blot was probed with a fragment from the TbAATP6 gene and a ß tubulin gene was used as a control. Lanes 1-4 indicate days post tet induction (T0, 0 hr; T1, 24 hr; T2, 48 hr; T3, 72 hr).

Northern analysis of RNA from ΔTbAATP6-i (Figure 3-18) shows that the target TbAATP6 mRNA was readily detectable in transfected cells in the absence of the inducer tetracycline (Lane 1: T0). However, analysis indicated that degradation of the TbAATP6 mRNA was not efficient. At time points up to 72 hours no loss of mRNA was seen, with only a significant loss of mRNA after 24 hours (Lane 2: T1). This means that RNAi gene silencing of TbAATP6 is not operating in an efficient and inducible manner in this instance. RNAi is powerful tool for selective interference of gene expression in PCF but experiments presented here didn't demonstrate its effectiveness.

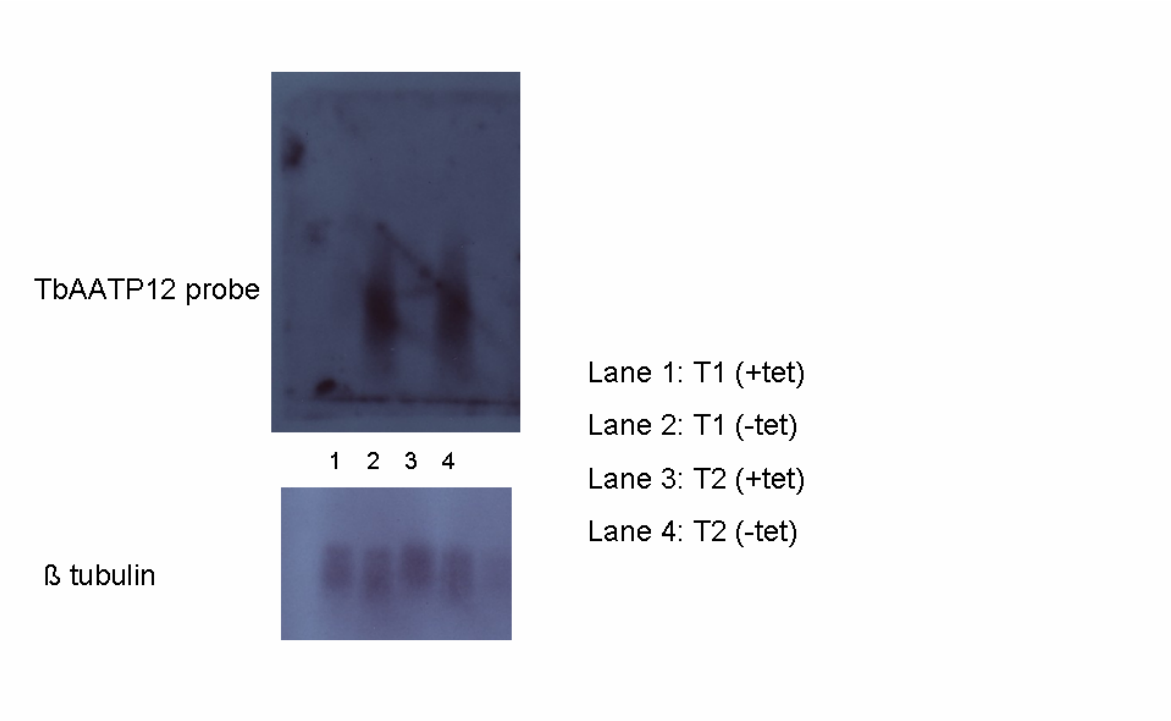


Figure 3-19: Northern blot analysis of RNA from TbAATP12 RNAi cell lines.
Northern blot was probed with a fragment from the TbAATP12 gene and a ß tubulin gene was used as a control. Lanes 1-4 indicate days post tet induction.

Northern analysis shows that the target TbAATP12 mRNA was readily detectable in transfected cells in the absence of the inducer tetracycline and that degradation of the TbAATP12 mRNA was efficient, even after 24 hours post tetracycline induction (Figure 3-19).

3.2.7 Tetracycline Induction and Growth Curves

The growth of clonal cells carrying the RNAi construct upon tetracycline induction was compared uninduced cells. Growth was determined in normal medium as well as in medium containing single carbon sources (SDM80 plus proline or glucose at 10 mM). In ΔTbAAT6-i PCF clones, tetracycline induction caused a dramatic growth phenotype, not only in SDM79, but also in SDM80 supplemented with 10 mM L-proline. Growth of induced cell lines in SDM80 plus D-glucose (10 mM) showed no difference from uninduced cell lines.

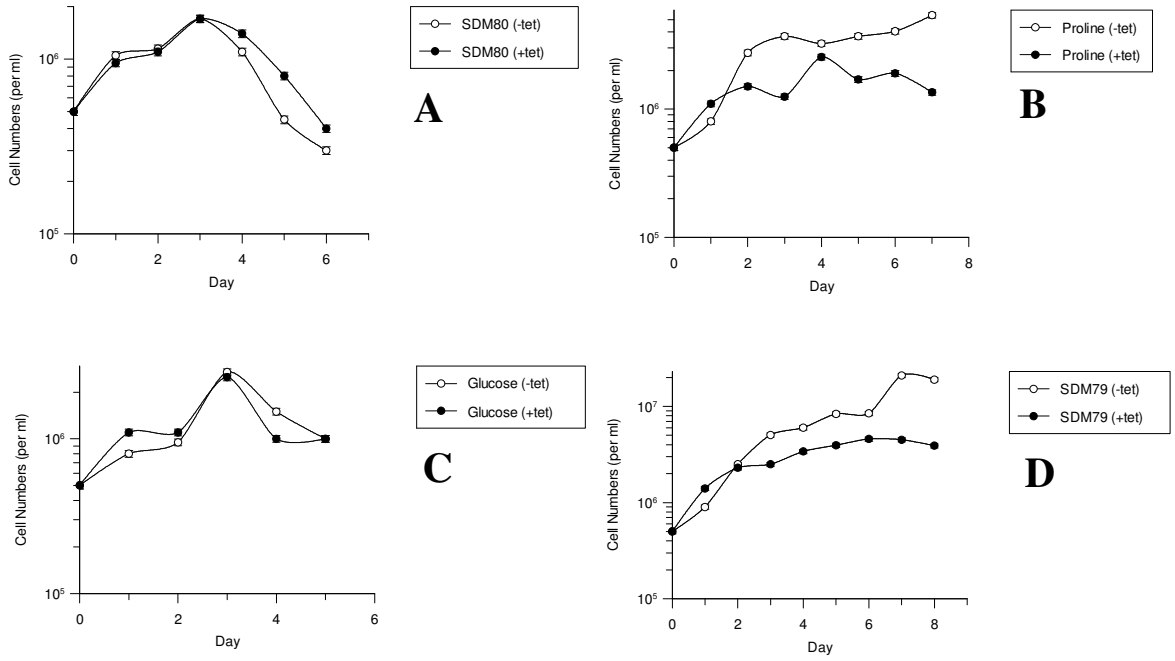


Figure 3-20: Growth of TbAATP6 RNAi cell lines.
Tetracycline induced and uninduced cell lines grown in SDM80 (panel A), SDM80 plus 10 mM L-proline (panel B), SDM80 plus 10 mM D-glucose (panel C) and SDM79 medium (panel D) (this result represents data from 3 similar repeats).

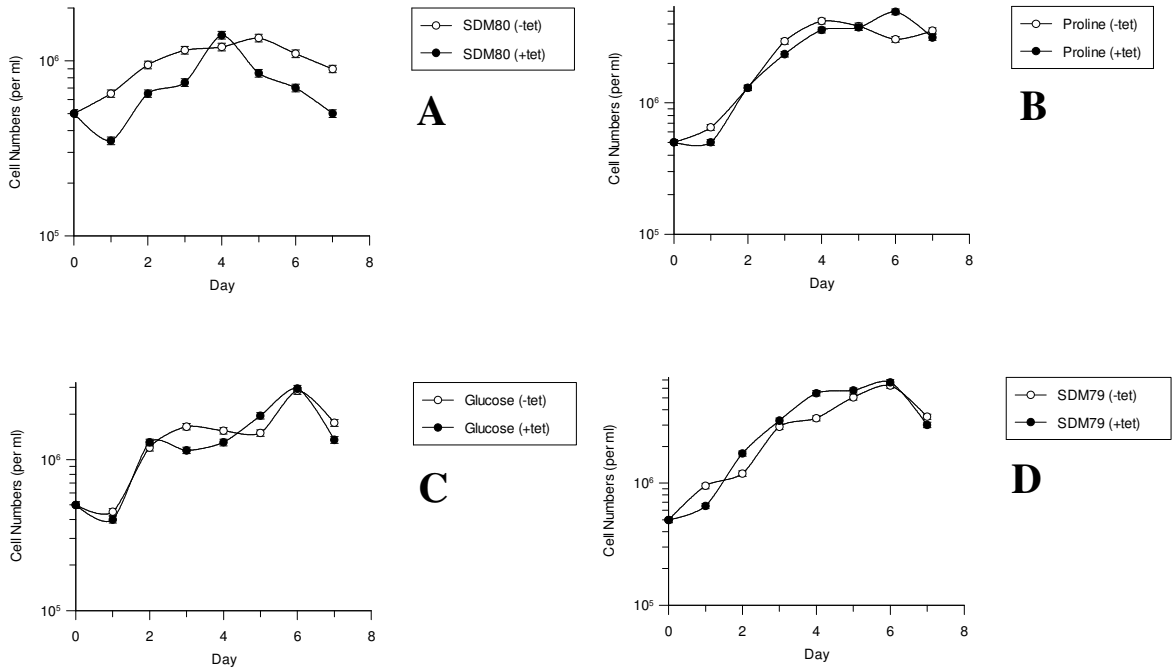


Figure 3-21: Growth of TbAATP12 RNAi cell lines.
Tetracycline induced and uninduced cell lines grown in SDM80 (panel A), SDM80 plus 10 mM L-proline (panel B), SDM80 plus 10 mM D-glucose (panel C) and SDM79 medium (panel D) (this result represents data from 3 similar repeats).

For ΔTbAATP6-i, a decrease in the growth rate of induced culture in SDM79 medium was evident within 24 hours of tetracycline induction. The uninduced cells grew at much higher rate than the induced cells (Figure 3-20 D), exhibiting a reduced doubling time in normal medium and proline only medium. Microscopic inspection of the cells showed no major changes in cell morphology upon the addition of tetracycline. L-proline is the only amino acid that can serve as a carbon source (Lamour *et al.*, 2005). It is possible that TbAATP6 has a role in proline transport due to the fact that a reduction of growth upon tetracycline induction is associated with growing cells in proline only medium. However, with the northern blot analysis showing ineffective knock-down of the gene transcript, the significance of this is most likely be due to abarrent effects of the RNAi mechanism and not due to the TbAATP6 gene.

In ΔTbAATP12-i PCF clones, tetracycline induction caused no significant growth phenotype. Growth of ΔTbAATP12-i induced cell lines in SDM80 plus D-glucose (10 mM), SDM79, SDM80 plus L-proline (10 mM) showed no difference from uninduced cell lines. Moreover, microscopic inspection of the cells showed no major changes in cell morphology upon the addition of tetracycline.

3.2.8 TbAATP6 Transporter Phenotype

I wanted to test a possible transporter phenotype to see if TbAATP6 knock-down corresponds to reduced uptake of a particular amino acid. Since TbAATP6 showed a growth phenotype, particularly in proline only medium, I assessed TbAATP6 as a possible proline transporter.

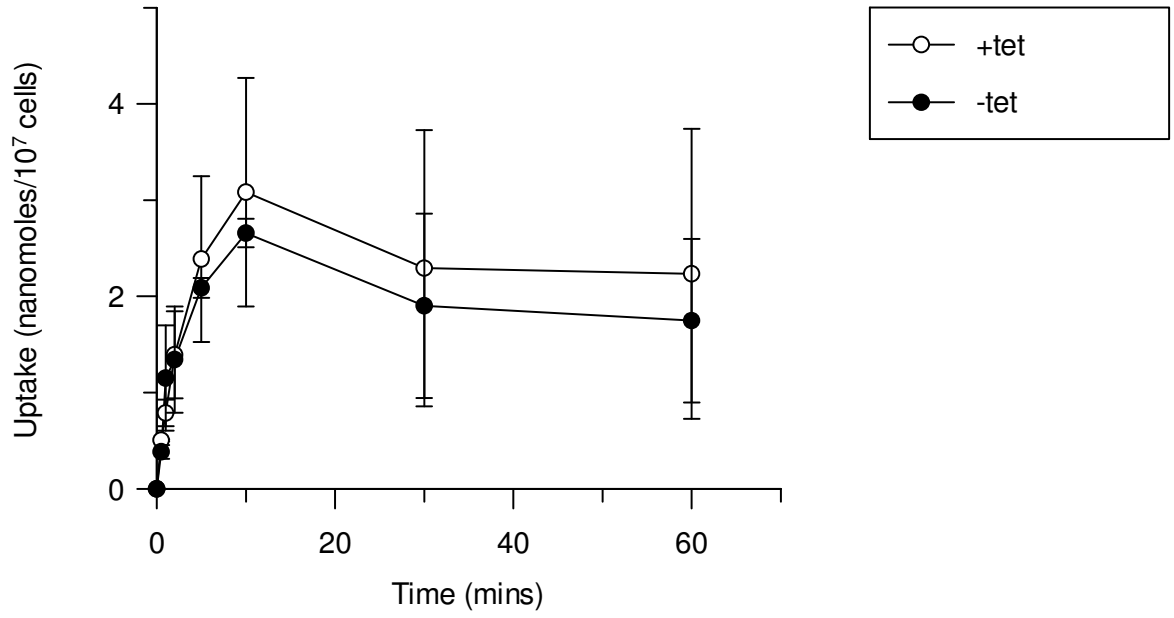


Figure 3-22: Proline uptake in TbAATP6 RNAi cell lines.
Uptake of 100 µM L-[^{3}H] Proline in TbAATP6 RNAi cell lines (plus and minus tet induction) over a 1 hour time period (n=3, Error bars = ±SEM).

TbAATP6 RNAi cell lines showed no reduced accumulation of proline over the one hour time period, suggesting that this transporter is not involved in proline uptake. However, in the absence of a working RNAi mechanism (as shown by northern blots) a phenotype for TbAATP6 cannot be postulated.

3.3 Discussion

Why the redundancy in amino acid transporter genes? It is possible that in addition to the fact that genes correspond to a given/particular structure-activity relationship, amino acid transporter genes could have evolved out of a particular need for a particular metabolic pathway. It is possible that important metabolites involved in the same pathway could be co-transported together, resulting in different transporters for different amino acid groups, of which the groups are not based on structural relationships.

3.3.1 TbAATP1

Previous studies involving the use of a number of amino acids to competitively inhibit the uptake of others have allowed the differentiation of several separate amino acid transporters (Voorheis, 1971). Four amino acid transport systems were defined; first neutral amino acid carrier (transporting glycine, alanine, serine and threonine), second

neutral amino acid carrier (transporting valine, leucine, proline and methionine), first acid amino acid carrier (transporting glutamate only), and the first basic amino acid carrier (transporting lysine only). However, it is now evident from the vast number of amino acid transport genes identified from the genome database as well as the biochemical characterisations of transport of certain amino acids that a more complex story is at play.

Voorheis (1971) reported the existence of a neutral amino acid transport system in bloodstream form trypanosomes (questionable since no data was presented). Here I report, through RNAi, a similar transport system that exists in PCF trypanosomes. TbAATP1 (AAT7) mediates the transport of alanine, threonine, serine, glycine, cysteine and asparagine. This genomic locus seems unique to *T. brucei* as no homeologs are found in *Leishmania* or *T. cruzi*. This suggests that only *T. brucei* has a transporter system unique for the amino acids transported by TbAATP1 (ser, gly, cys, ala, asn, thr).

Acetate derived from threonine has a role as a precursor for lipid biosynthesis and inhibition of threonine metabolism via the enzyme aldehyde dehydrogenase showed a marked growth phenotype (Voorheis *et al.*, 1975). Here, with TbAATP1, I show some indication to a growth phenotype when downregulation of this transporter system is in play. However, this growth phenotype has not been linked exclusively to reduced threonine uptake.

The segment of TbAATP1 amplified for creation of the RNAi construct shows high sequence homology to the three unannotated genes flanking TbAATP1 (termed TbAATP2). TbAATP1 and TbAATP2 show >80% sequence identity (with 70% usually needed for knock down experiments). Therefore, it is a certainty that the RNAi phenotype is also as a result of TbAATP2 knock down. Most amino acid transporters have been biochemically characterised but few have been related to a specific genomic locus. Here, uptake of L-serine, glycine, L-cysteine, L-asparagine, L-alanine and L-threonine is specifically linked to the AAT7 genomic locus of *T. brucei*. This genomic locus seems to play a role in cell growth as seen by stunted growth of cell lines exposed to tetracycline for prolonged periods. However, this growth phenotype does not seem to be related to immediate transporter activity, yet seems more of a delayed effect of initial amino acid depletion.

I have shown an order of affinity for the TbAATP1 transport system (L-threonine>>L-serine>>Glycine>>L-alanine>>L-cysteine>>L-asparagine). Does the order of preference relate to downstream metabolic requirement? Only few studies into amino acid usage in

trypanosomes have been performed, and most focus on a few. Apart from use in protein synthesis the most biologically relevant of the TbAATP1 specific amino acids are L-threonine and L-cysteine. Cysteine is an essential growth factor for bloodstream form trypanosomes and is involved in protein biosynthesis as well as in the production of glutathione and trypanothione (Duszenko *et al.*, 1992). Dr I. Accoceberry has suggested at least one other route of entry for L-cysteine (unlabelled cysteine was unable to inhibit radiolabelled cysteine).

L-threonine usage by African trypanosomes has previously been studied and been found to be a precursor for acetate (which feeds into lipid biosynthesis) in *T. brucei* (Cross *et al.*, 1975). There is cleavage of L-threonine to form glycine and acetate within the mitochondrial compartment. Preliminary examination of changes in medium amino acid levels during growth of *Trypanosoma brucei* revealed complete metabolisation of L-threonine (Cross *et al.*, 1975). BSF and PCF trypanosomes were reported previously to be able to catabolise L-threonine (Colasante *et al.*, 2006). In PCF trypanosomes threonine metabolism has been shown to occur exclusively in the mitochondrion: here threonine is converted to acetyl-CoA and glycine by the enzymes L-threonine 3-dehydrogenase (EC 1.1.1.103) and acetyl-CoA:glycine C-acetyltransferase (Opperdoes *et al.*, 1981).

Here I have shown no essentiality for L-threonine in PCF trypanosomes. Removal of threonine from the growth medium showed no disadvantage for growth of wild type cells whether proline or glucose was used as the primary carbon source. This suggests that the acetate units provided by acetyl-CoA from the catabolism of threonine can be compensated for by another source (pyruvate when glucose is metabolised and fatty acid metabolism when glucose is absent). It is also possible that the internal threonine pool can be supplemented by another source. Lack of threonine essentiality suggests that the growth defect of ΔTbAATP1-i cell lines is not as a result of threonine alone and most likely due to the other TbAATP1 specific amino acids, either individually or cumumulatively. Salvage of these six amino acids in trypanosomes occurs via transport processes as well as from side products of other metabolic processes with the exception of glycine and serine, of which biosynthesis does not seem to occur from another metabolic pathway (KEGG). The essentiality of all AATP1 specific amino acids (individually and in combination) will need to be investigated much in the same way threonine was investigated.

The six amino acids specific for TbAATP1 give more insight into the structural specificity of the transporter. Asparagine is the only TbAATP1 specific amino acid with a hydrophilic side group and, consequently, is the least specific for TbAATP1. However, its side group is

small enough to be recognised by the transporter. Other amino acids with hydrophilic side groups contain a charged group or are too large to be recognised by the transporter. The rest of the TbAATP1 specific amino acids share common neutrality and a small side chain of two carbon units or less.

There is a consistency shown among the uptake of currently characterised amino acids by their various systems. Modes of entry of amino acids have affinities in the micro molar range, reflecting the relative abundance of these substances the parasite encounters. Here I have determined the kinetic constants of threonine transport by ΔTbAATP1-i cell lines. A K_m value of 73.925 μM and a V_{max} of 21.18 nanomols/min/10^8 cells were calculated. L-methionine transport in both PCF and BSF parasites were measured in the presence of various methionine concentrations (Hasne & Barrett, 2000). The kinetic constants were determined by non-linear regression analysis using the Michaelis–Menten equation. A K_m value of 30.99 +/-7.6 μM and a V_{max} of 16.29 +/- 0.01 nmol/min/10^8 cells were calculated. For uptake in bloodstream forms, a K_m value of 32.89 +/-3.4 μM and a V_{max} of 28.89 +/- 0.1 nmol/min/10^8 cells were calculated (Hasne & Barrett, 2000). Proline uptake in procyclic trypanosomes has been shown to be carrier mediated with an apparent K_m of 21 +/-2.9 μM and a V_{max} of 7.0 nanomoles/min/10^8 cells. This level of uptake is modulated by the presence or absence of glucose in the growth media (Lamour *et al.*, 2005).

Cell survival is dependent on the ability of cells to maintain a chemically stable intracellular milieu (for optimal enzyme activity) and to regulate their volume. Cells exposed to a medium of reduced osmolarity swell initially, but subsequently undergo compensatory shrinkage by a regulatory volume decrease (RVD) response. Different types of osmolytes have been implicated in the RVD process, from amino acids such as alanine, glutamine, glycine, proline, and glutamate, to sugars (Viera *et al.*, 1998). The ability of *Leishmania* to regulate its volume has been studied. When *L. donovani* are challenged with an acute decrease in osmolarity, the parasites rapidly release amino acids of which Ala, the major osmolytes in cells, accounted for half of the total amino acid loss (Darling & Blum, 1990).

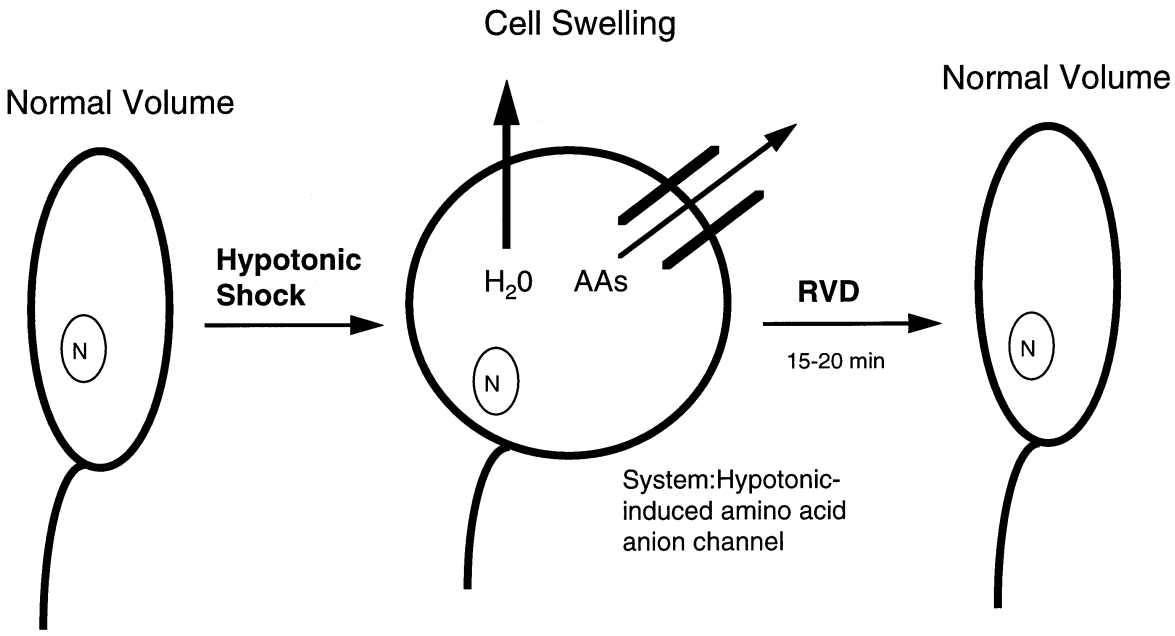


Figure 3-23: Volume regulation in trypanosomatid parasites under hypotonic shock (taken from Viera *et al.*, 1998).
AAs, amino acids (neutral and negatively charged); N, nucleus; RVD, regulatory volume decrease.

Here, I have shown the TbAATP1 transporter system not to be implicated in osmoregulation. However, other systems could be at play. It is entirely possible that the length of time needed for ΔTbAATP1-i induced cells to recover resting shape within hypotonic media is altered. Hence, no difference in swell shape is seen between both cell lines.

The kinetics of release of amino acids from the intracellular pool can be explored much in the way it has been previously (Voorheis & Martin, 1980). Cells previously incubated with a metabolically inert amino acid (2-amino [1-^{14}C] isobutyric acid) were then exposed to varying osmotic strengths, with the release of radioactivity measured over time.

The amino acid analogue glycine chloromethyl ketone (1-amino 3-chloropropan-2-one) has been shown to act as a specific irreversible inhibitor of the neutral amino acid transport system (Owen & Voorheis, 1975). Pre-incubation of cells with the analogue and then subjecting them to hypotonic stress would be a way to ascertain if this transport system is involved in osmoregulation.

3.3.2 TbAATP6 & TbAATP12

The RNAi technique was used to study the physiological role of two other genes (TbAATP6 and TbAATP12) in *T. brucei* PCF trypanosomes. Elimination of transcripts in cell lines transfected with the P2T7Ti vector carrying a fragment of the AATP gene was evaluated by Northern blot analysis. With TbAATP6 significant degradation of transcript was not seen in tetracycline induced cell lines up to 72 hours post induction. With TbAATP12 loss of mRNA was seen as early as 24 hours post induction. Lack of an effective RNAi mechanism at work in ΔTbAATP6-i cell lines could be as a result of sequence similarity to a related AATP gene. Tb11.01.7500 and Tb11.01.7520 (AAT16 and AAT17) are the closest related AATP and show a ~50% sequence identity to TbAATP6. That identity is spread throughout the gene sequence and not limited to a particular part of the open reading frame. It is possible that the RNAi mechanism, in this case is not operating in a gene specific manner. The RNAi construct created for TbAATP6 takes a gene fragment from the C-terminal end of the protein (3' end of the open reading frame). With RNAi sometimes C-terminal RNAi targeting is ineffective (Dr. Alibu, personal communication) and most of the time, N-terminal targeting works out better. Hence, for efficient targeting of TbAATP6, a gene fragment towards the N-terminal of the gene and one that will provide less crosstalk between related gene products is needed in the design of future RNAi experiments.

The fact that ΔTbAATP6-i cell lines showed reduced growth in normal medium and proline only medium suggested a possible role for the transporter associated with proline metabolism. Proline uptake in tetracycline induced cell lines showed no significant difference from uninduced cell lines. However, this experiment was performed after 72 hours tetracycline induction; a time shown by Northern analysis to have no significant degradation of mRNA transcript. It is possible that cross-reaction of the RNAi mechanism to another related gene results in stable down-regulation of that transcript. However, with no reduction in proline uptake we must assume that neither TbAATP6 nor a related similar AATP acts as a proline transporter. Another possibility exists; one that involves another amino acid or group of amino acids that are under regulation by glucose. Proline regulation by glucose availability has been shown (Lamour *et al.*, 2005; chapter 4). Increased reliance of other amino acids in the absence of glucose is a possibility that has yet to be explored. It is important to note that any suggested phenotype is inadmissible in the absence of a working RNAi mechanism.

TbAATP12 RNAi cell lines showed no growth defect with tetracycline induction regardless of primary carbon source in the growth medium, ruling out a role for TbAATP12 in cell growth. A transport phenotype still remains to be determined for this cell line. Uptake assays using all 20 radiolabelled amino acids will need to be performed in order to ascertain if uptake of any amino acid is effected.

4 Proline Transport Regulation as a Result of a Metabolic Shift Induced by N-Acetyl D-Glucosamine

4.1 Introduction

The *T. brucei* lifecycle is complex, and the parasite adapts to life in both its insect host (tsetse fly) and in the mammalian hosts. These distinct environments require the parasite to remodel its metabolism to thrive within each (Besteiro *et al.*, 2005). In the D-glucose-rich environment of the mammalian bloodstream the parasite relies solely on D-glucose to produce energy. D-glucose can be metabolised by either the glycolytic pathway or the pentose phosphate pathway (Barrett, 1997). The first seven steps of glycolysis occur sequestered in the peroxisome-like organelle, the glycosome (Opperdoes & Borst, 1977; Michels *et al.*, 2006).

In PCF trypanosomes, the mitochondrion has traditionally been held to produce the major proportion of the cell's ATP (Bochud-Allemann & Schneider, 2002). This is largely due to the use of alternative carbon sources like L-proline as the input for mitochondrial metabolism (Besteiro *et al.*, 2005). ATP can be generated by oxidative phosphorylation (electron transport chain), or by substrate-level phosphorylation, with an unusual reaction catalysed by succinyl-CoA synthase, and coupled to acetate formation being key (Bochud-Allemann & Schneider, 2002; van Hellemond *et al.*, 1998; Rivière *et al.*, 2005). Recently, the exact contribution of the Kreb's cycle to energy generation has been looked at in detail (Van Weelden *et al.*, 2003; Van Weelden *et al.*, 2005), and it appears that in spite of most Kreb's cycle enzymes being present, the flux of substrates through these enzymes does not occur in the classical sense. However, these studies – as with many related to procyclic metabolism – were performed on trypanosomes grown in D-glucose-rich medium. D-glucose apparently represses L-proline metabolism in procyclic trypanosomes (Lamour *et al.*, 2005; Evans & Brown, 1972). In these conditions the parasite appears capable of fulfilling energy needs without using the Kreb's cycle or electron transport chain and by producing ATP primarily by substrate level phosphorylation (oxidative phosphorylation is not essential).

In the mammalian host, trypanosomes divide as long slender bloodstream forms, covered by a dense coat of variant surface glycoproteins (VSG) (Cross, 1975; Roditi, 1996). When ingested by the fly in the blood meal, the short stumpy bloodstream forms which are cell-cycle arrested and are pre-adapted to life within the tsetse fly (Roditi *et al.*, 1989), differentiate into procyclic form trypanosomes that are able to colonise the midgut of the fly (Roditi *et al.*, 1989). Parasite differentiation is accompanied by the replacement of the VSG coat by an invariant coat of EP or GPEET procyclins (Roditi *et al.*, 1989; Roditi *et*

al., 1998). EP expression continues throughout the fly stage of the procyclic form whereas GPEET expression is downregulated in late procyclics (Vassella *et al.*, 2000; Acosta-Serrano *et al.*, 2001). In culture, however, GPEET expression can be modulated by glycerol or D-glucose (Vassella *et al.*, 2000). RNAi silencing of the first step of glycolysis, hexokinase, caused a dramatic switch from EP-procyclins to GPEET-procyclin expressed on the outer coat (Morris *et al.*, 2002); suggesting that removal of D-glucose enhances GPEET-procyclin expression. Regulation of expression of EP- and GPEET-procyclin isoforms appears to be controlled by mitochondrial enzymes of the acetate:succinate CoA-transferase (ASCT) cycle (Vassella *et al.*, 2004). Silencing of the hexose transporter and the phosphofructokinase enzyme also upregulated GPEET-procyclin expression, indicating that a reduction in glycolytic flux correlates with EP to GPEET switch.

L-proline metabolism too is modulated by D-glucose availability (Lamour *et al.*, 2005). Whether one or more metabolites act to trigger such a switch is not known. For instance, it was proposed that D-glucose (or its analogue 2-DOG) alone was capable of inducing the repression of L-proline metabolism (Lamour *et al.*, 2005), since the presence of the non-metabolisable analogue (2-DOG) is sufficient to block growth of parasites using proline as an energy source. It is possible that 2-DOG affects cells in a way that is independent of a down-regulation of proline metabolism when glucose is absent. However, it is also possible that glucose and 2-DOG act allosterically to bring about proline down-regulation.

In addition to profound differences between BSF and PCF trypanosomes in glucose metabolism, uptake of this carbohydrate also differs in BSF and PCF *Trypanosoma brucei brucei*. Different transporter isoforms make the major contribution to uptake. The Trypanosome Hexose Transporter 1 (THT1) is expressed in bloodstream forms at a level 40 times that of THT2, while procyclic form trypanosomes express only the THT2 isoform (Bringaud *et al.*, 1992; Barrett *et al.*, 1995). The C2 substituents of D-glucose, D-glucosamine (GlcN) and N-acetyl D-glucosamine (GlcNAc) have previously been shown to interact with the hexose transporter of bloodstream form trypanosomes (Eisenthal *et al.*, 1989). D-glucose and GlcN uptake has been shown. However, in the case of GlcNAc there was no apparent uptake in bloodstream forms (Azema *et al.*, 2004) although an interaction with the hexose transporter was noted, since GlcNAc inhibits uptake of 2-DOG and GlcN. The ability of PCF to accumulate GlcNAc has not been studied.

Establishment of trypanosome infection is as complicated in the fly as it is in the mammalian system. The parasite must establish itself as a dividing procyclic population before development can progress. Natural infection rates are low and attributed to many

environmental and intrinsic fly factors (Welburn & Maudlin, 1999; Dale & Welburn, 2001; Welburn *et al.*, 1993). Like other insects, the tsetse fly has an effective innate immune system and several components, including midgut lectins, have been implicated in trypanosome killing, playing a role in determining vector susceptibility (Welburn *et al.*, 1989; Welburn & Maudlin, 1990). Lectins, a class of proteins that bind certain carbohydrates noncovalently, found in the midgut of *Glossina morsitans* (Ibrahim *et al.*, 1984) mediate the lysis of trypanosomes and at the same time have been postulated to provide the signal for the parasites to mature (Welburn & Maudlin, 1989). The role of lectins in preventing trypanosome infection was inferred from enhanced infection rates after feeding the lectin-inhibitory amino sugars GlcN and GlcNAc. The amino sugars GlcN and GlcNAc have multiple effects in establishing parasite midgut infection. Both sugars occur naturally in the gut of some flies through the action of chitinase produced by the non-obligatory endosymbiont *Sodalis glossinidius* (Dale & Welburn, 2001). The sugars block lectin-mediated trypanosome killing as well as inhibiting trypsin activity (Osir *et al.*, 1993, Abubakar *et al.*, 2006). This is proposed to underlie the increase in infection rates (Mihok *et al.*, 1992). Recently, it was proposed that these sugars may have anti-oxidant function that aids in establishing infection (Macleod *et al.*, 2007).

It has been shown that GlcNAc also stimulated trypanosome growth *in vivo* in the fly gut and also *in vitro* in the absence of fly derived factors (Peacock *et al.*, 2006). We have investigated the underlying basis for this effect. Given that bloodstream form trypanosomes do not show any apparent uptake of GlcNAc (Azema *et al.*, 2004), we aimed here to determine whether procyclic forms could accumulate GlcNAc, or whether its effects might be due to other mechanisms. For example, either as a simple direct growth factor or alternatively, through inhibition of D-glucose uptake leading to an induced metabolic shift that enhances growth.

Repression of proline catabolism seems to be directed at both the transporter and the proline dehydrogenase enzyme (PRODH). Differential expression of amino acid transporters, as a result of the duality of the life cycle of these parasites, is expected and requires that changes in its surrounding environment be observed in the different life cycle stages. Within individual life cycle stages we also get differential expression of amino acid transporters as a result of differing extracellular conditions. Proline regulation as a function of glucose availability has been shown in PCF trypanosomes (Lamour *et al.*, 2005). It is possible that regulation of other amino acid transporters occur as a result of glucose or other factors, whether in PCF or BSF trypanosomes; and this regulation might play a role in the development of the parasite.

4.2 Results

4.2.1 GlcNAc Stimulates Growth of PCF Trypanosomes

GlcNAc has previously been show to stimulate growth of procyclic form trypanosomes (strain J10) *in vitro* using Cunningham's medium (CM) (Peacock *et al.*, 2006). To determine whether a similar effect was seen using other *T. brucei* strains we investigated growth of *T. brucei* strain TREU 927.

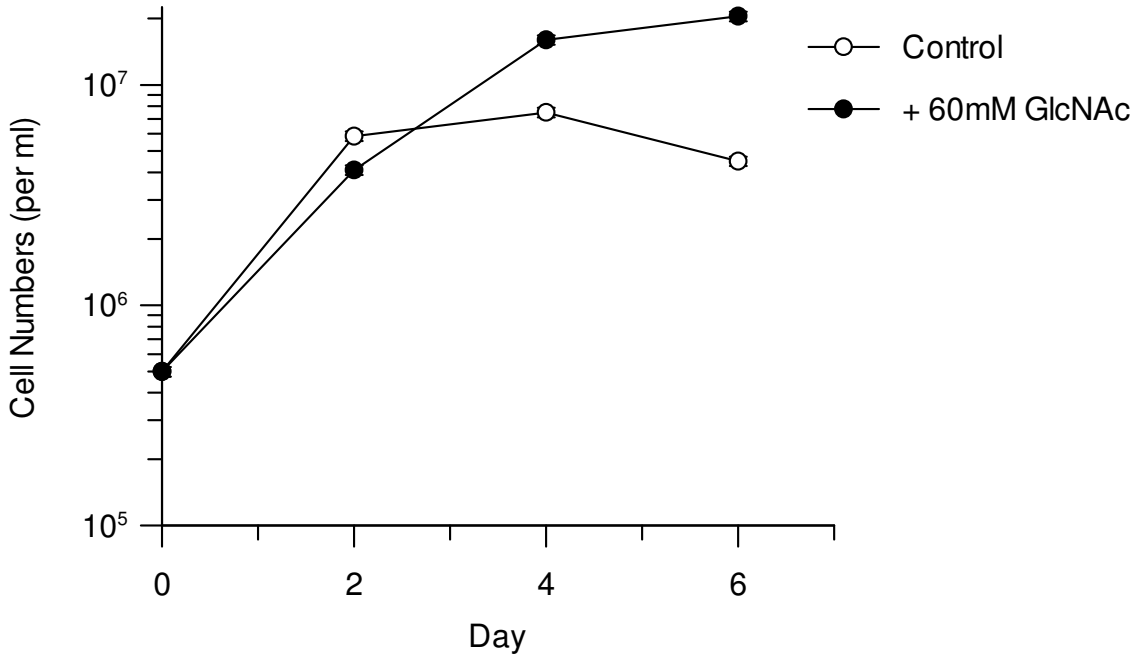


Figure 4-1: Cell counts of PCF trypanosomes (strain TREU 927).
A starting density of 5 x 10^5 cells per ml, grown in CM and CM supplemented with 60 mM GlcNAc (this represents data from 3 similar repeats, error bars = ±SEM).

60 mM GlcNAc enhanced survival and increased final density, with an increase in growth rate after 2 days (Figure 4-1). GlcNAc enhancement of growth could be as a direct result of its usage as a potent energy source. To test whether GlcNAc itself was capable of supporting growth, we tested the ability of trypanosomes to grow in medium containing this amino sugar but no D-glucose or L-proline (SDM80, Appendix 8.2).

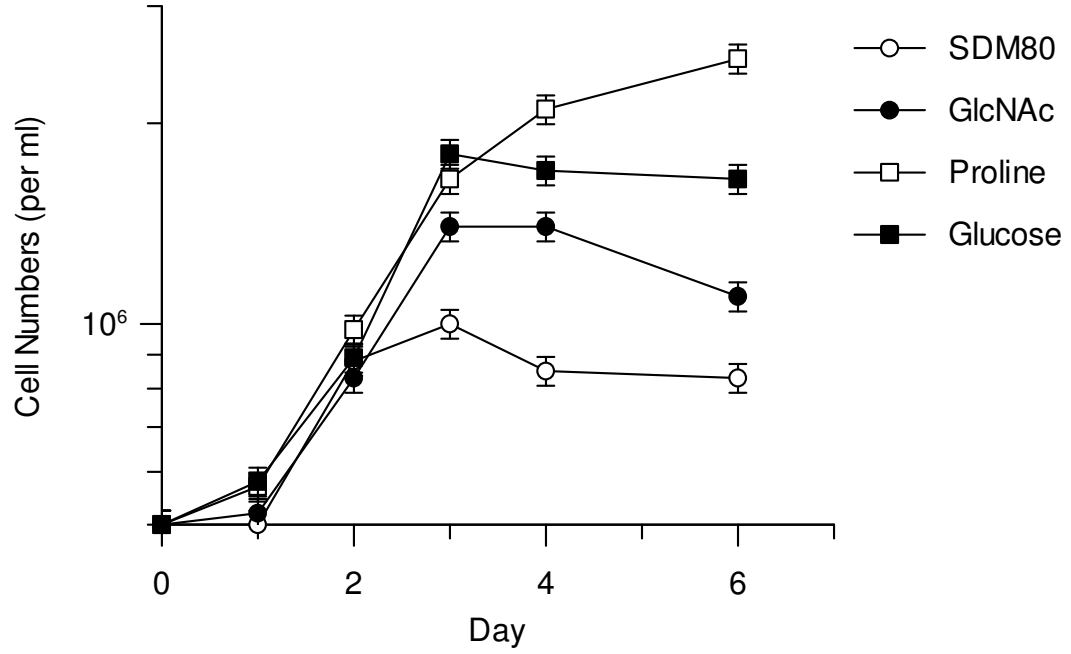


Figure 4-2: Cell counts of PCF trypanosomes (strain TREU 927) grown in SDM80 medium. A starting density of 5 x 10^5 cells per ml, grown in SDM80 (D-glucose- L-proline-depleted medium) supplemented independently with 60 mM GlcNAc, 10 mM L-Proline, 10 mM D-Glucose or 10 mM L-Proline and 10 mM D-Glucose (this represents data from 3 similar repeats, error bars = ±SEM).

While either L-proline or D-glucose supported robust growth, GlcNAc failed to do so, ruling out a role for GlcNAc as an energy source for these parasites (Figure 4-2). L-proline metabolism too is modulated by D-glucose availability (Lamour *et al.*, 2005). Glucose is the preferred carbon source and down-regulates proline metabolism. It is possible that non-internalised GlcNAc prevents glucose utilisation by blocking of the hexose transporter, thereby releasing proline from inhibition, resulting in a metabolic switch from glucose metabolism to proline metabolism.

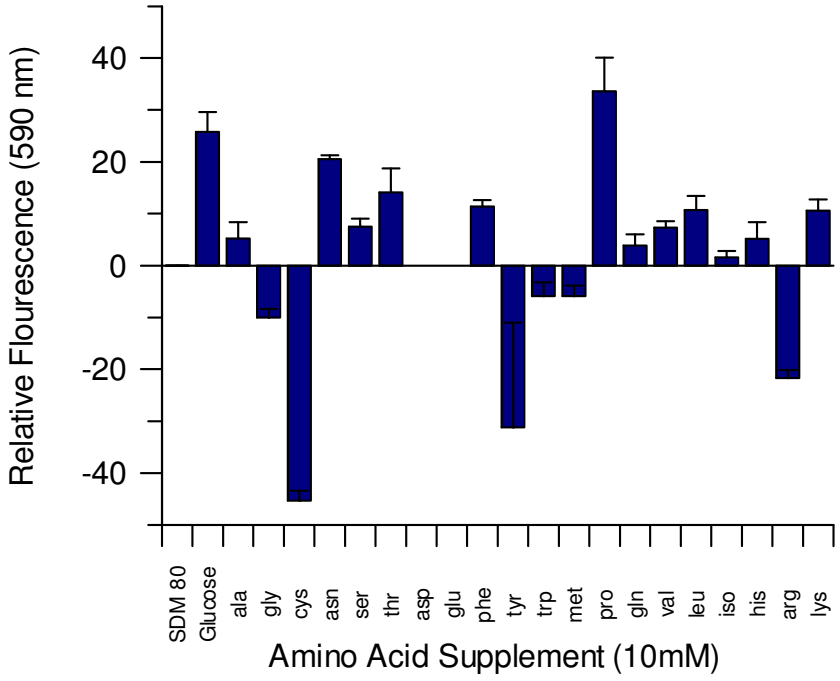


Figure 4-3: Alamar Blue assay using PCF trypanosomes grown in SDM80.
SDM80 medium supplemented with 10 mM of each of the 20 amino acids and D-glucose individually (n=3, error bars = ±SEM).

The Alamar blue assay (Figure 4-3) shows that proline as principal carbon source yields a more significant growth of trypanosomes than glucose or any other amino acid (data not shown for the amino acids aspartate and glutamate). The apparent increase in fluorescence seen by the amino acids glutamate and aspartate is anomalous, as the non metabolised D-isomers of these amino acids do not support growth as seen from the cell count data (Figure 4-4). It is possible that the acidic nature of these amino acids interferes with the resazurin giving a false positive. The Alamar Blue method is relatively insensitive when trying to assess the exact level of growth exhibited. To assess the exact nature of the increased growth stimulus provided by proline over glucose cell counts were done.

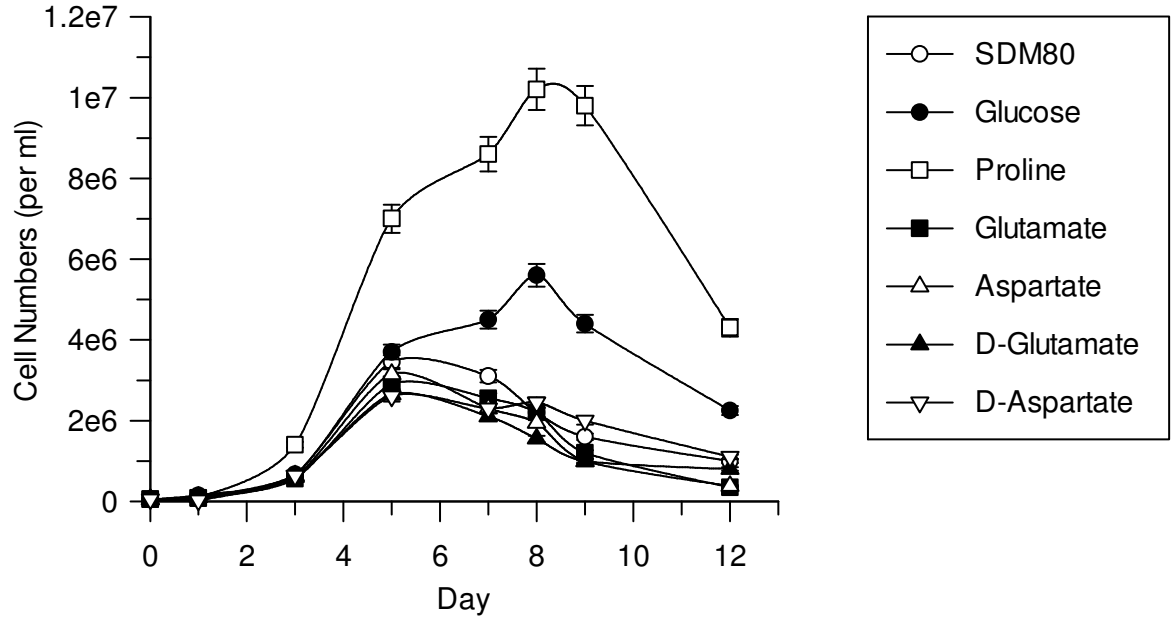


Figure 4-4: Cell counts of PCF trypanosomes (strain TREU 927) grown in SDM80.
A starting density of 5 x 10^5 cells per ml, grown in SDM80 (D-glucose- L-proline-depleted medium) supplemented independently with D-glucose, L-proline, L-glutamate, L-aspartate, D-glutamate, D-aspartate at 10 mM concentrations (this represents data from 3 similar repeats, error bars = ±SEM).

As previously reported, the D-glucose and L-proline-depleted medium (SDM80) fails to support robust growth of procyclic form trypanosomes (Lamour *et al.*, 2005). Supplementation with L-proline or with D-glucose allows growth, while other amino acids do not. In fact, L-proline supports more robust growth than D-glucose (Figure 4-4).

4.2.2 GlcNAc is not Internalised by PCF Trypanosomes

To verify further its inability to act as a carbon source, we investigated GlcNAc as a possible substrate for the PCF hexose transporter. Interaction of GlcNAc with the hexose transporter without uptake has previously been reported in BSF trypanosomes (Azema *et al.*, 2004) and we investigated whether this was also the case in PCF trypanosomes. The ability of the procyclic hexose transporter to recognise GlcNAc and GlcN was assessed by determining the ability of each to inhibit uptake of radiolabelled 2-DOG (2-DOG has previously been shown to be a suitable probe to study D-glucose uptake in procyclic form trypanosomes, Barrett *et al.*, 1995).

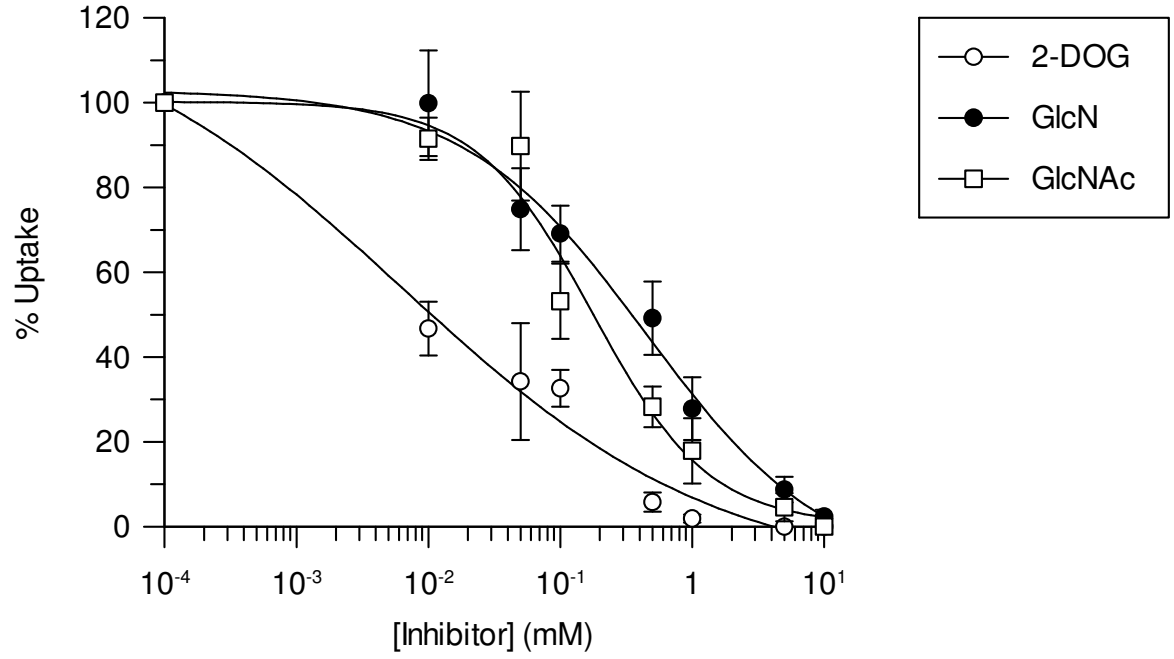


Figure 4-5: 2-Deoxy-D-glucose inhibition.
Inhibition of uptake of 10 µM [6-^{14}C] 2-DOG with cold GlcNAc (open squares), GlcN (closed circles) and 2-DOG (open circles) over a range of concentrations (10 µM, 50 µM, 100 µM, 500 µM, 1 mM, 5 mM, 10 mM). IC_{50} values were calculated for each inhibitor (n=3, error bars = ±SEM).

IC_{50} values (Table 1) were calculated for each inhibitor (2-DOG = 16.5 µM ± 9.3, GlcNAc = 176.18 µM ± 65.28, GlcN = 443.96 µM ± 18.39). This suggests that GlcNAc and GlcN do exhibit interaction with the procyclic hexose transporter, although they show a relatively low affinity (10- and 30-fold lower than 2-DOG, respectively).

Inhibitor	IC_{50} (µM)	SEM
2-Deoxy-D-glucose	16.5	9.3
Glucosamine	443.96	18.39
N-Acetyl D-Glucosamine	176.18	65.28

Table 4: Calculated IC_{50} values for analogue inhibitors.

However, interaction with the transporter does not necessarily mean internalisation of the analogue, and accumulation of the analogues was tested using uptake assays of radiolabelled sugars (Figure 4-6).

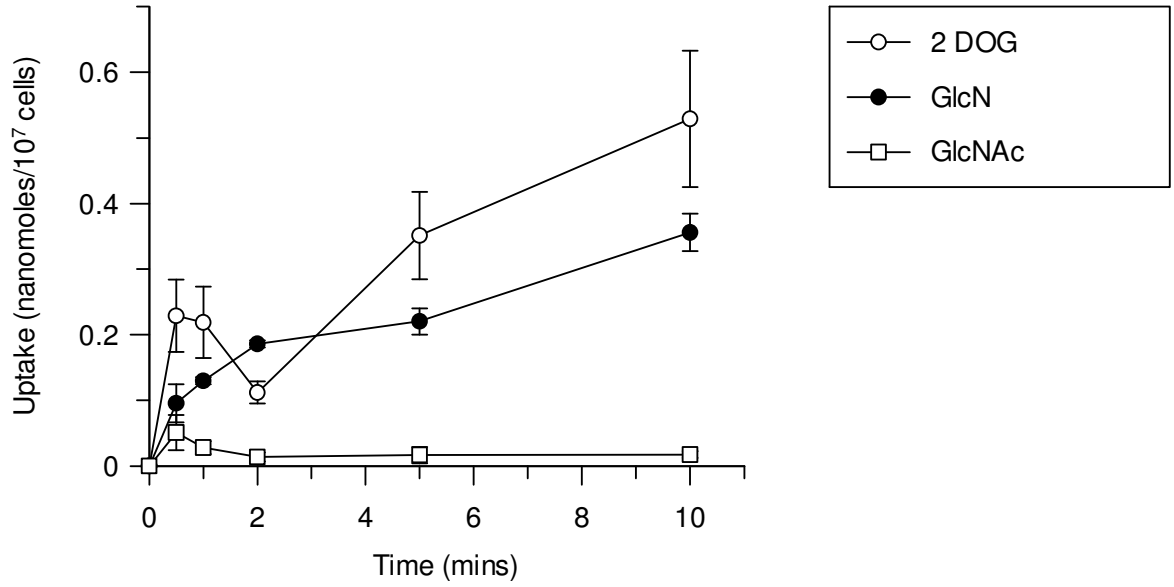


Figure 4-6: Uptake of Glucose analogues.
Uptake of 1 mM [6-^{14}C] -2-DOG (open circles), [1-^{14}C] GlcN and [^{3}H] GlcNAc (n=3, error bars = ±SEM).

As previously shown with the bloodstream form hexose transporter, there was no internalisation of GlcNAc into PCF trypanosomes. In comparison, the D-glucose analogues 2-DOG and GlcN were both recognised and internalised by the parasite. Thus GlcNAc neither acts as a carbon source nor enters trypanosomes, although it does interact with the hexose transporter and thereby inhibits D-glucose uptake, by competing with the transporter.

4.2.3 Presence of GlcNAc Causes a Switch to Proline Metabolism

GlcNAc inhibits D-glucose uptake without itself being internalised by PCF trypanosomes. To address the question as to whether the effect of GlcNAc is related to its capacity to diminish D-glucose uptake and to induce a metabolic shift we analysed the D-glucose present in the medium after 24 hrs during log phase growth of trypanosomes (strain TREU 927). Glucose is quantified spectrophotometrically using the Glucose (GO) Assay Kit (Sigma). The kit employs enzymatic reactions to produce a coloured end-product, proportional to the original glucose concentration, which can be measured at 540 nm. Briefly, D-glucose is oxidized to gluconic acid and hydrogen peroxide by glucose oxidase, with hydrogen peroxide reacting with *o*-dianisidine in the presence of peroxidase to form a coloured product. Oxidized *o*-dianisidine reacts with sulfuric acid to form the measurable end-product

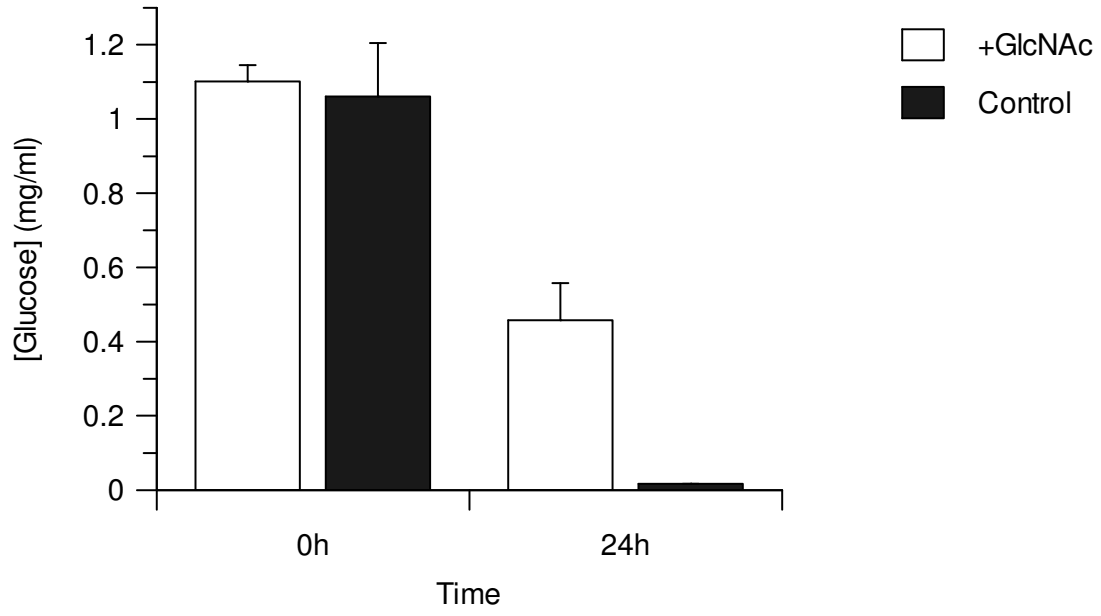


Figure 4-7: Glucose utilisation in presence of GlcNAc.
Residual D-glucose concentration in culture medium during log phase growth of procyclic form trypanosomes (strain 927) in the presence and absence of GlcNAc (n=3, error bars = ±SEM).

With GlcNAc present in the medium, D-glucose is consumed at a lower rate by the parasites (Figure 4-7). The quantity of L-proline consumed by the procyclic trypanosomes has previously been shown to be decreased when D-glucose is available as the principal carbon source (Lamour *et al.*, 2005). To assess if GlcNAc supplemented in the medium can stimulate this switch to increase proline utilisation, PCF trypanosomes were grown in Cunningham's medium (approx. 4 mM D-glucose and 60 mM L-proline; Appendix 8.4) plus or minus GlcNAc (60 mM), SDM80 supplemented with D-glucose (10 mM), or L-proline (10 mM) and then assayed for L-proline uptake.

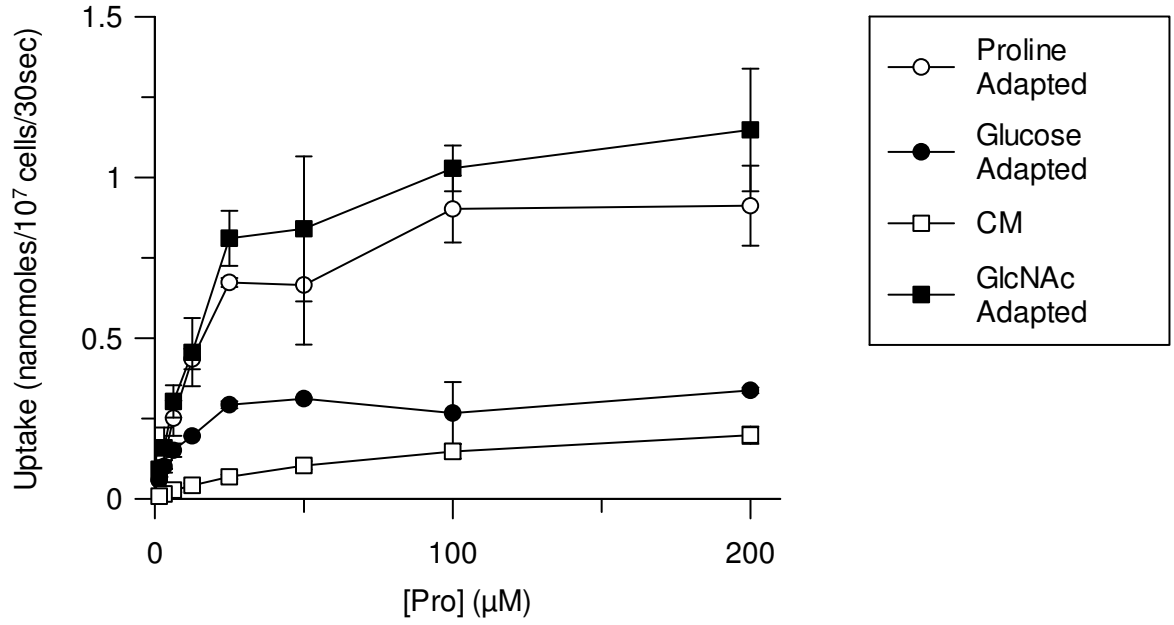


Figure 4-8: L-proline uptake.
Procyclic form trypanosomes pre-adapted to different medium concentrations and then assayed for L-proline uptake. Uptake of [^{3}H] L-proline into procyclic (strain TREU 927) trypanosomes grown in Cunningham's medium (CM containing 4 mM D-glucose and 60 mM L-proline) (white square), SDM80 supplemented with 10 mM D-glucose (black circles), SDM80 supplemented with 10 mM L-proline (white circles) and CM supplemented with 60 mM GlcNAc (black squares). Uptake was measured at a single 30 s time point over a range of concentrations (up to 200 µM) (n=3, error bars = ±SEM).

Pre-adaptation to D-glucose deficient conditions (in SDM80 based medium) increased L-proline uptake in procyclic form trypanosomes, to a rate twice that in cells grown in high D-glucose conditions (Figure 4-8). Cells grown in CM showed an even further reduction in L-proline uptake. However, addition of GlcNAc to the medium induced L-proline uptake to levels comparable to cells grown in high L-proline medium. It thus appears that the presence of GlcNAc induced increased L-proline consumption, probably as a consequence of its capacity to diminish uptake of D-glucose.

4.2.4 Oligomycin Sensitivity

Oligomycin is the most sensitive known inhibitor of the F_0/F_1 ATPase enzyme, which is critical in ATP generation using the mitochondrial respiratory chain, but not when cells produce ATP by substrate level phosphorylation. It has been reported that PCF trypanosomes growing in a D-glucose-rich media are over 1000 times less sensitive to oligomycin than parasites growing in D-glucose-depleted medium (Coustou *et al.*, 2003; Lamour *et al.*, 2005). Oligomycin at a concentration of 0.1 ng/ml has been shown to kill all

cells utilising proline as the principal carbon source whereas with glucose that value increases to 10 µg/ml.

A modified Alamar Blue method was employed, where procyclic trypanosomes (strain TREU 927) were grown in the presence of oligomycin (0.1 ng/ml) and L-proline (5 mM) and the D-glucose concentration was titred in doubling dilutions (5 mM starting concentration).

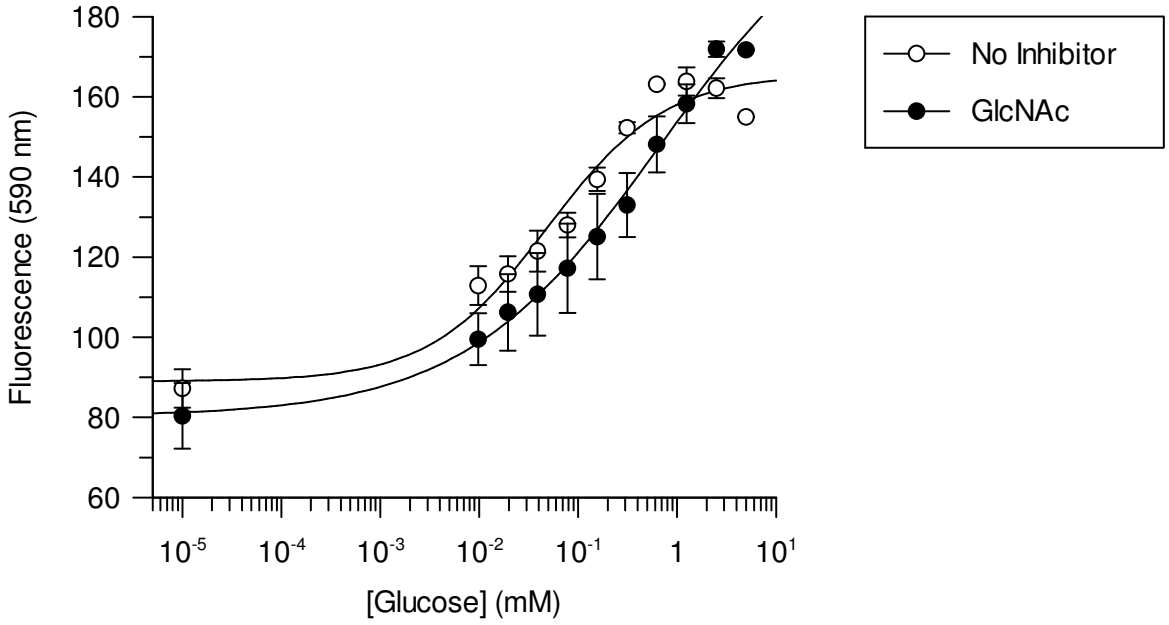


Figure 4-9: Oligomycin sensitivity.
Alamar Blue of PCF trypanosomes exposed to 0.1 ng/ml oligomycin in the presence of 5 mM proline and decreasing concentrations of glucose (5 mM starting concentration), in the presence and absence of GlcNAc (n=3, error bars = ±SEM).

The D-glucose IC_{50} value for oligomycin sensitivity significantly increases by more than ten fold from 48.6 µM to 674.5 µM in the presence of GlcNAc. The metabolic switch appears to be a graded response, with oligomycin sensitivity increasing as D-glucose levels decrease, indicating a tightly regulated balance. From this, it is evident that, the same link between D-glucose availability, L-proline consumption and oligomycin sensitivity is observed.

4.2.5 GlcNAc Protects Against Death by 2-Deoxy D-glucose

The non-metabolised glucose analogue 2-DOG has been shown to lead to the death of PCF trypanosomes using proline as the sole carbon source (Lamour *et al.*, 2005; Kessler & Parsons, 2005). This killing effect was suggested to be related to the repression of proline

usage that glucose (and 2-DOG) exerts (Lamour *et al.*, 2005). This effect of 2-DOG is ameliorated by increasing concentrations of glucose in the media (Kessler & Parsons, 2005). Here we examine quantitatively how 2-DOG exerts an effect on proline utilisation, and whether GlcNAc offers any protection to the toxic effects of 2-DOG.

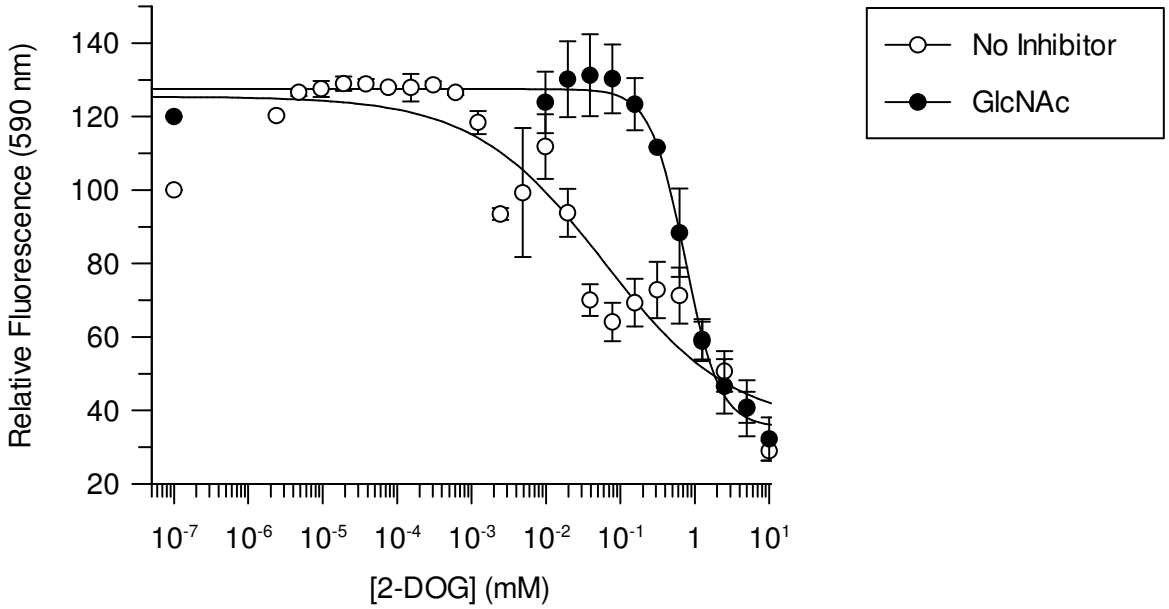


Figure 4-10: 2-Deoxy-D-glucose sensitivity.
Alamar Blue of procyclic form trypanosomes grown in the presence of 5 mM L-proline and decreasing concentrations of 2-DOG (10 mM starting concentration), in the presence and absence of GlcNAc (n=3, error bars = ±SEM).

The effects of 2-DOG are dependent on concentration. Limiting concentrations of 2-DOG give an IC_{50} of 93.9 µM. GlcNAc at a concentration of 60 mM is able to protect against the effect of 2-DOG, due to its capacity to diminish 2-DOG entering the cell, significantly increasing the IC_{50} to 953.2 µM.

4.2.6 Proline Regulation as a Result of the Overall Metabolic Status of the Cell

In PCF trypanosome proline consumption is tightly linked to glucose availability. Proline consumption as a function of glucose concentration shows a dose dependent response to glucose, indicating a gradual replacement of glucose metabolism by proline metabolism – rather than an "on-off" switch stimulated by glucose reaching a critical limiting concentration.

The ability of these parasites to adapt to glucose depleted conditions may be altered by long-term *in vitro* culturing in glucose rich medium (Bringaud *et al.*, 2006). The general lab strains *T. brucei* PCF 427 and *T. brucei* PCF 927 have generally been cultured in standard high glucose medium (SDM79, Cunningham's Medium). We posed the question as to how long is necessary for cells to be exposed to glucose for it to exert its repression on proline metabolism? Does glucose entering the cell exert repression directly? To answer this question cells were grown in high proline, no glucose and then assayed for proline uptake over a 6 hour period in the presence or absence of 10 mM glucose.

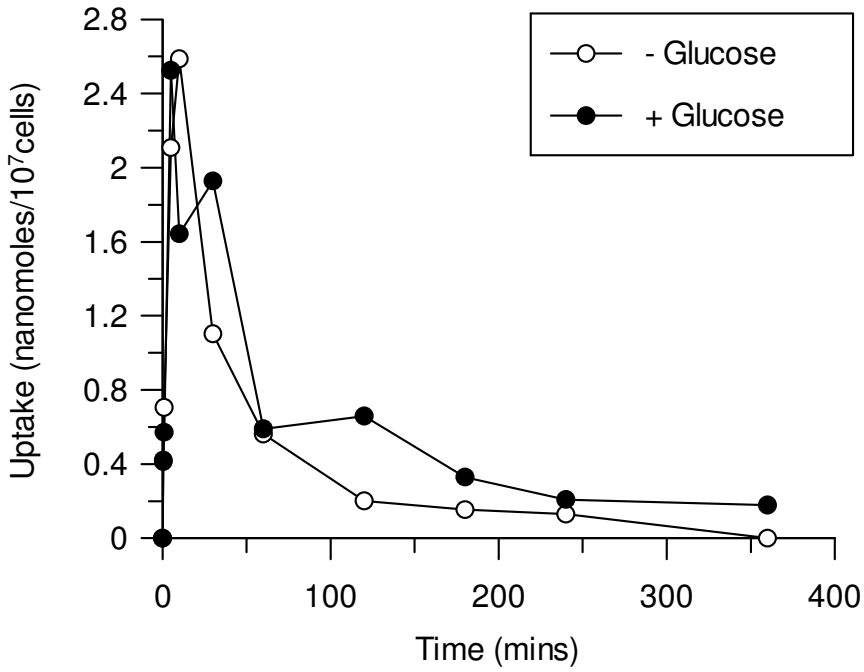


Figure 4-11: Proline uptake in presence of D-glucose.
Uptake of 100 µM [^{3}H] L-Proline in proline adapted cell lines in the absence (white circles) and presence (black circles) of 10 mM Glucose, over a 6 hour time period (n=1).

There seems to be no difference in proline uptake between proline adapted cells in the presence and absence of 10 mM glucose. Proline uptake reaches a maximum point at 5 – 10 mins, with apparent efflux after that. This suggests that glucose repression is not a result of glucose itself, but rather the adaptational processes that result from glucose availability. This also suggests that proline regulation is probably not regulated at the glucose transporter level. Because if simple entry of glucose into the cell was all that was required to reduce the uptake of proline we would expect to see reduced consumption of proline over time. This all gives more weight to glycolysis and glycolytic enzymes in control of metabolic adaptation.

However, this does not tell us how much time is needed for glucose to exert its effect, only that the glucose entering the cell is not immediately exerting its repression over proline. To answer the time dependency question, we adapted PCF trypanosomes to proline only conditions by growing in SDM80 plus 10 mM proline. At time zero we split cell cultures and added 10 mM glucose to one of the flasks and assayed for proline uptake, at a single 30 second time point, over a 24 hour period.

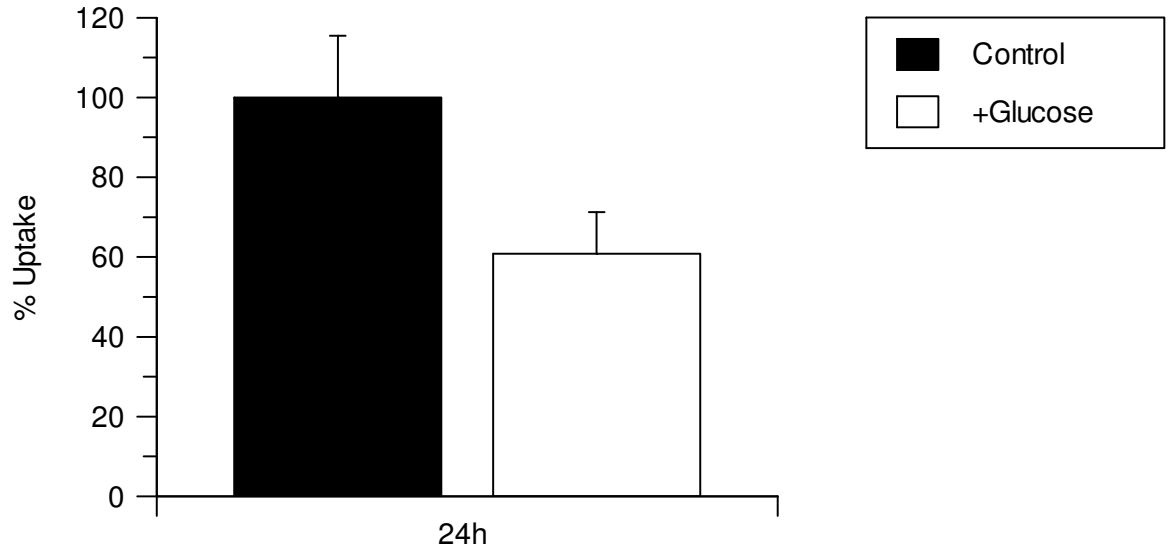


Figure 4-12: Proline uptake after long term D-glucose exposure.
Uptake of 100 µM [^{3}H] L-Proline in PCF trypanosomes after a 24 hour period exposure to 10 mM D-glucose (n=3, error bars = ±SEM).

It is evident that after 24 hours in the presence of D-glucose proline uptake repression is beginning to take effect. Proline uptake shows a ~40% reduction after 24 hours in the presence of D-glucose. This, together with the previous experiment on proline uptake in the presence of D-glucose in the assay buffer shows that the repressive effects of D-glucose on proline are not as a direct result of the entering glucose. Yet, it seems the entire metabolic status of the cell (i.e. glycolytic flux) is more likely to be the determinant for proline repression.

4.3 Discussion

It has been long established that GlcNAc enhances colonisation of tsetse by trypanosomes, which has been causally linked to GlcNAc inhibition of midgut trypanocidal lectins (Abubakar *et al.*, 2006; Mihok *et al.*, 1992; Osir *et al.*, 1993; Welburn & Maudlin, 1999). The demonstration that GlcNAc also stimulates growth *in vitro*, in the absence of any fly-

derived factors was surprising (Peacock *et al.*, 2006). With the fact that GlcNAc is not internalised by BSF trypanosomes (Azema *et al.*, 2004) and D-glucose exerts repressive effects on proline consumption we investigated the possibility that the in vitro effects of GlcNAc was brought about by the ability of the analogue to block glucose entry to the cell via the hexose transporter, thereby resulting in a switch to L-proline metabolism, which provides a better growth effect.

We show here that the stimulation of growth is not as a direct result of GlcNAc utilisation as a carbon source, because GlcNAc is not internalised by the parasite and cannot replace D-glucose or L-proline as a carbon source for energy. However, there is interaction with the hexose transporter, demonstrated by GlcNAc inhibition of uptake of 2-DOG.

We therefore propose that GlcNAc, through blocking of the procyclic hexose transporter, promotes a metabolic shift in procyclic form trypanosomes leading to an increased consumption of L-proline, as previously noted when procyclic form trypanosomes were grown in glucose-depleted media (Lamour *et al.*, 2005). L-proline metabolism in PCF trypanosomes is more efficient than D-glucose metabolism. When D-glucose and L-proline are both present in the growth medium, however, the procyclic form trypanosome will preferentially use D-glucose as the main carbon source (Bringaud *et al.*, 2006; Cross *et al.*, 1975).

It appears, therefore, that the *in vitro* growth stimulatory effect of GlcNAc relates to the switch from D-glucose to L-proline catabolism, leading to an increase in L-proline consumption. This is correlated to increased oligomycin sensitivity. The switch is a graded response, rather than a binary distinction, since L-proline consumption is gradually reduced as D-glucose levels rise, and there is a corresponding gradual decrease in susceptibility to oligomycin. The repressive effect of D-glucose on L-proline metabolism seems to be related to the overall metabolic status of the cell rather than any allosteric action of D-glucose on its own, since cells adapted to growth without D-glucose retain high levels of L-proline uptake rates for many hours even when D-glucose is added.

This up-regulation of proline consumption seems to be at the transporter and enzymatic level (PRODH enzyme; Lamour *et al.*, 2005). Whether one or more metabolites act to trigger such a switch is not known, however, the switch is as a result of glucose metabolism (down stream metabolites). It is possible that D-glucose itself does not act allosterically to bring about the transporter repression, suggesting that a time factor for

adaptation exists. It is evident that the cell up-regulates proline transport. Whether this up-regulation involves a single transporter or several transporters is still to be elucidated.

In spite of the metabolic shift that accompanies growth in L-proline, procyclics using L-proline as the main carbon source show no infectivity advantage to tsetse flies over control procyclics grown in normal D-glucose-rich medium (W.C. Gibson, personal communication). This suggests that the *in vivo* effects of GlcNAc are not a result of any metabolic advantage of the trypanosomes, but rather are a consequence of reduction in fly anti-microbial defences such as inhibition of lectins (Welburn & Maudlin, 1999).

In addition to its impact on L-proline catabolism, D-glucose exerts an influence on other aspects of procyclic trypanosome biology, including procyclin expression as well as mitochondrial energy generation (Butikofer *et al.*, 1997; Morris *et al.*, 2002; Vassella *et al.*, 2001). The reason for the negative regulation exerted by D-glucose on L-proline metabolism is far from clear, especially in the light of the fact that L-proline provides a markedly better growth stimulus than D-glucose. A possible explanation could be linked to the developmental cues associated with reduced D-glucose availability. When BSF trypanosomes are taken up by the tsetse vector, presumably glucose availability drops rapidly during digestion of the bloodmeal in the tsetse midgut. It is possible that D-glucose availability is one marker involved in life cycle progression of the parasite. It will therefore be of interest to determine whether the response of procyclics to D-glucose with regard to L-proline catabolism is linked to the response in expression of particular procyclin surface membrane protein isoforms or is related to processes leading to developmental differentiation of the parasite.

5 Metabolomics

5.1 Introduction

It is evident that PCF trypanosomes exhibit metabolic adaptational processes (Lamour *et al.*, 2005; chapter 4). We have shown that a switch to proline metabolism occurs (as indicated by oligomycin sensitivity and proline uptake) (chapter 4). This switch is a graded response to glucose availability and can be mimicked by blocking of the PCF hexose transporter by GlcNAc. However, what is not known is how far reaching this metabolic switch goes. It is our intent to investigate, through metabolic profiling, what other markers emerge as a result of this metabolic shift.

Metabolic changes, as a result of differing primary carbon source usage detected in the Orbitrap visible metabolome will be particularly informative when comparing with previous studies using these targeted approaches to investigate parts of the metabolome. With this in mind, we decided to investigate the metabolome of PCF trypanosomes with regard to the metabolic adaptation as a result of differing carbon source usage.

Systems biology is a rapidly emerging field. Technological advances have allowed metabolomics, the study of all metabolites within a given system, to emerge as a research area (Breitling *et al.*, 2006). Individual metabolites, and their relative abundance within cells can be assessed by a range of techniques (Breitling *et al.*, 2006; Goodacre, 2004; Dettmer *et al.*, 2007), one of which involves Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) (Brown *et al.*, 2005; Hirai *et al.*, 2004; Marshall *et al.*, 1998). Fourier transform mass spectrometry determines the mass-to-charge ratio (m/z) of ions based on the cyclotron frequency of the ions in a fixed magnetic field. The signal (free induction decay) detected as an image current is transformed to its mass spectrum using the Fourier equation.

The recently introduced Orbitrap mass analyser (Hu *et al.*, 2005) offers resolution and accuracy close to that of classical high field FT-ICR-MS or FTMS (Breitling *et al.*, 2006). However, the Orbitrap operates using electrostatic rather than magnetic fields and hence by-passes the requirement of a large, super-cooled magnet. This allows it to be linked to chromatographic columns and thus avoids the risk of ion suppression effects which can occur with directly infused complex samples. This technique can resolve thousands of metabolites, in the molecular mass range of 100–1500, with extreme mass accuracy (routinely <2 ppm). This technique has been employed to build *ab initio* networks of metabolites whose masses can be linked by common biochemical transformations in trypanosomes (Breitling *et al.*, 2006). Exact mass can also be used to predict likely

formulae for metabolites and tentatively to identify compounds based on comparison to databases of known metabolites. The fact that multiple formulae can yield masses within 2 ppm (Kind & Fiehn, 2006) means that different approaches are also required to confirm identity.

In FTMS (including Orbitrap analysis), ions, generated from soft ionisation methods (e.g. electrospray) are excited into cyclotron resonances that are a function of mass and charge. The frequency of gyration of these ions is detected in the mass spectrometer and then converted to high accuracy masses using the Fourier transform. The determined mass is as a result of several different physical principles within the analyser (Hu *et al.*, 2005). The ionised metabolite mixture is trapped in an orbital trajectory of which the frequency of the orbit depends on mass/charge ratio.

In the case of the orbitrap, the trapping is achieved without the need of a magnetic field, with ions trapped in a radial electric field between a central and outer cylindrical electrode. The ultra-high resolution enables simultaneous identification of ions that would not be resolved using less accurate mass determination approaches. The combined advantage of accurate mass determination and resoultion makes it possible to restrict the number of possible molecular formulae that represent an individual molecular mass (Hu *et al.*, 2005; Breitling *et al.*, 2006).

The number of metabolites present in trypanosomes is sufficiently small to ensure very good resolution using high resolution, high mass accuracy mass spectrometry without the need for subcellular fractionation. Metabolomics requires special approaches for sample preparation, separation, and mass spectrometric analysis (Dettmer *et al.*, 2007). The metabolome represents a vast number of components belonging to a wide variety of classes of compounds (amino acids, lipids, organic acids, nucleotides, etc). Such diversity of compounds have reflecting diversity in their physical and chemical properties and occur in a wide range of abundances. To this effect, a careful experimental design is needed for the success of metabolomic investigations (Dettmer *et al.*, 2007). While processing samples, formation and degradation of metabolites due to remaining enzyme activity or oxidation processes is a possibility (Dettmer *et al.*, 2007). Therefore, rapid quenching and metabolism inhibition is required. Rapid quenching of metabolism with hot HEPES-buffered ethanol with subsequent freezing in liquid nitrogen has proven an effective method of identifying metabolites in our lab (D. Wildridge, personal communication). Any and every kind of preparation step will involve metabolite loss (Dettmer *et al.*, 2007). For

example, aqueous extraction results in poor recovery of very polar compounds as well as the loss of volatile organic compounds (Dettmer *et al.*, 2007).

Within the field of metabolomics three distinct types of investigation exist; metabolic profiling, metabolic fingerprinting and metabolic footprinting (Dettmer *et al.*, 2007). Profiling involves quantitative targeted metabolite analysis of metabolites of a specific class or related to a selected pathway. The disadvantage of profiling is the fact that targeted analysis is a hypothesis-driven approach rather than a hypothesis-generating one, and that the data generated is not a global view of occurrences within the metabolome. Metabolic fingerprinting involves an unbiased global screening approach. The intention is not to identify each observed metabolite but allows comparison of patterns or "fingerprints" as a result of cellular perturbations. Fingerprinting observes the true phenotype, however, the causal metabolites of the observed phenotype must be identified if any biochemical understanding is to be gained from the data. This means that assignment of chemical formulae to masses must be made. The high resolution, unique to the Orbitrap processor, allows the potential to indentify large numbers of metabolites.

With this in mind, metabolomic fingerprinting can be used to classify or diagnose a particular perturbation. Metabolomic footprinting involves the analysis of extra-cellular metabolites in cell culture medium, which is a reflection of metabolites used or excreted by cellular processes. These three different metabolomic investigative methods shall be employed to address the question of metabolic adaptation in PCF trypanosomes.

Exact mass, used to predict likely formulae for metabolites, can be used to tentatively identify compounds based on comparison to databases of known metabolites (Kyoto Encyclopaedia of Genes and Genomes, www.genome.jp/kegg). Metabolic changes, as a result of differing primary carbon source usage detected in the Orbitrap visible metabolome will be particularly informative when compared with previous studies using these targeted approaches to investigate parts of the metabolome.

A complete representation of any cell, any organism, and any biosphere, which will enable computational prediction of higher-level complexity of cellular processes and organism behaviours from genomic and molecular information, is an area of research receiving much attention. Several widely-available databases have emerged to address this specific problem. KEGG is one of the most in-depth and comprehensive databases that exist for the identification of compounds and known metabolites. The KEGG pathway database is a collection of manually drawn pathway maps for metabolism and genetic information

(Kanehisa *et al.*, 2006). However, it is presently impossible to view global networks. To this end, several software packages have been developed for modelling of biomolecular interactions. An example is Cytoscape, which is an open source software package that allows the modelling of thousands of cellular components and interactions (www.cytoscape.org), either actual (*in vivo*) or hypothetical (*ab initio*). Dynamic states on molecules and molecular interactions are handled as attributes on nodes and edges (respectively), whereas static hierarchical data, such as protein-functional ontologies, are supported by use of annotations (Shannon *et al.*, 2003).

"MettaNetter" is a software plug-in developed for Cytoscape that allows inference and visualisation of the kind of high-resolution mass spectrometry data obtained directly from the Orbitrap processing (Fabien Jourdan, University of Toulouse). Inference requires a list of potential biochemical transformations, which are generated from commonly available biochemistry data as well as data sets from a range of databases, such as KEGG. The data extracted from the Orbitrap process is a list of masses (metabolites) and their corresponding intensities (qualitative). Each metabolite can be theoretically linked to another metabolite via a hypothetical biochemical transformation, and in turn more and more metabolites can be linked together; resulting in a global *ab initio* metabolomic network to be built (Figure 5-2). The Cytoscape program represents metabolites and their biochemical transformations as nodes and edges, respectively (Figure 5-1).

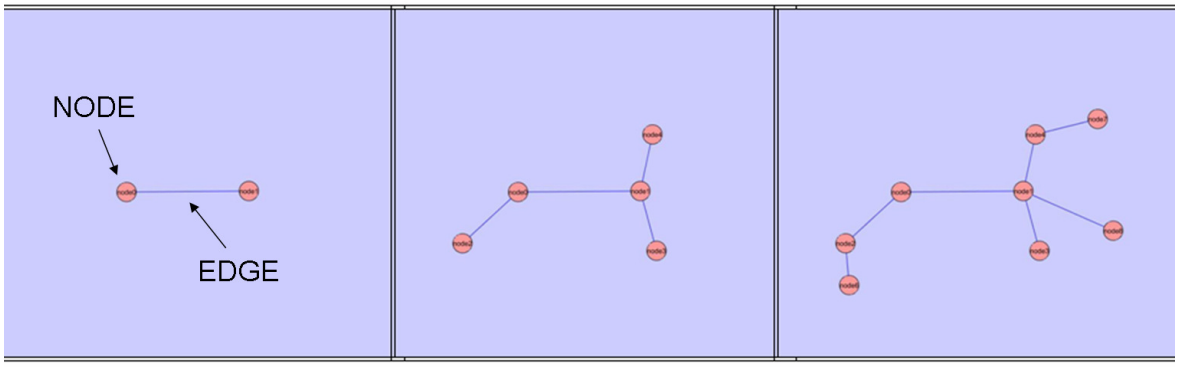


Figure 5-1: Nodes and edges.
Cytoscape representation of metabolites and biochemical interactions as nodes and edges, respectively. Nodes represented as circles and edges represented as joining lines.

The KEGG database contains approximately 488 trypanosome-specific metabolites and their resulting interactions (biochemical transformations). In collaboration with Fabien Jordan, their interactions and biochemical transformations were extracted to form a global

metabolomic network built using Cytoscape. The global network (Figure 5-2) is a representation of the different trypanosome pathways that exist within the KEGG database.

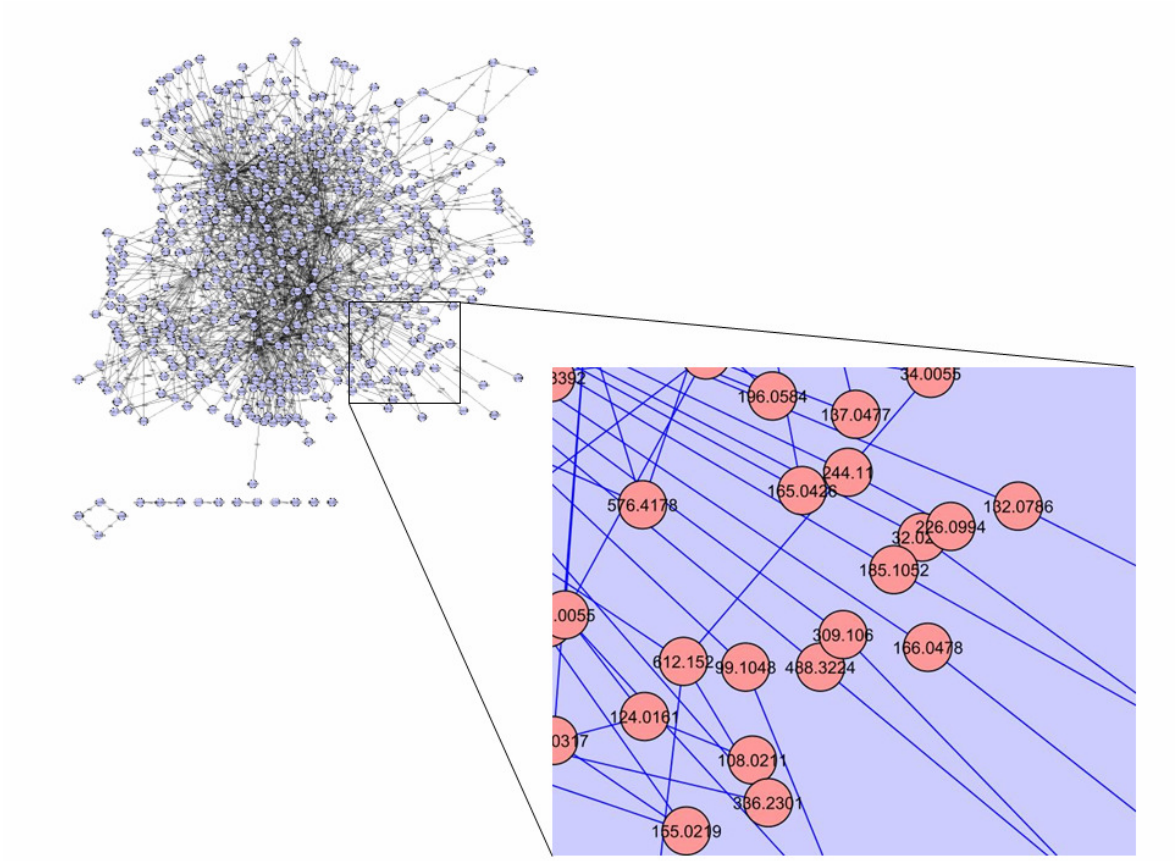


Figure 5-2: KEGG Network of *T. brucei* metabolites.
Cytoscape representation of trypanosomes specific metabolites from the KEGG database. Insert: shows particular metabolites and interactions in zoom.

The KEGG database contains 488 trypanosome specific metabolites and their resulting interactions (biochemical transformations). The global network (Figure 5-2) represents the inherent complementarity of the metabolic pathway within trypanosomes, and does not take into account any enzymatic data.

5.2 Results

5.2.1 Global View of the Metabolome

PCF trypanosomes were adapted to various conditions by growing in different medium for 7 days; Cunningham's medium (CM, Appendix 8.4), CM supplemented with 60 mM GlcNAc (NA), SDM80 medium (Appendix 8.2) supplemented with 10 mM D-glucose (GL), or 10 mM L-proline (PR). Parasites, at mid-log phase of growth, were harvested by

centrifugation (chapter 2.13) and resuspended in 1 ml of serum-free medium. Cell numbers attained were enough for 3 individual extractions. Cells were then incubated at room temperature for 30 mins. The intention being to allow metabolism to re-attain homeostasis but with a greatly concentrated cell density. Cells were separated from medium by spinning down at 6,000 rpm for 5 mins at 4ºC. 0.75 ml of 80% EtOH/20 mM HEPES (pH 7.0) was quickly added to separated medium and cell pellet, cooled on ice for 5 mins, vortexed, and then the supernatant recovered by centrifugation at 13,000 rpm for 5 mins. Recovered supernatant was frozen in liquid nitrogen and stored at -80ºC for further analysis.

Samples were processed using an Orbitrap MS (chapter 2.13). The Orbitrap mass spectrometry, employed here, is not quantitative (in the absence of an authenticated standard). However, the data generated is quantitative in a relative sense. The increase or decrease in size of a given peak is related to the changes in abundance of that metabolite across conditions. The exact masses derived from the Orbitrap mass spectrum are assigned putative formulae using an algorithm that sums all possible combinations of the elements C, H, O, P, N, S. Although for a given mass there may be many possible elemental combinations, in practice many of these can be eliminated as improbable.

Results presented here are from one culture subjected to 3 separate ethanol extractions. The final list of masses obtained from the Orbitrap mass spectrum were masses found in at least two of the three ethanol extractions.

The data extracted from the Orbitrap mass spectrum for each state was built into an *ab initio* metabolomic network. Metabolites, theoretically linked to other metabolites via a hypothetical biochemical transformation, using a list of common biochemical transformations (Appendix 8.16) within the mettanetter software allowed the global metabolome to be viewed for each metabolic state.

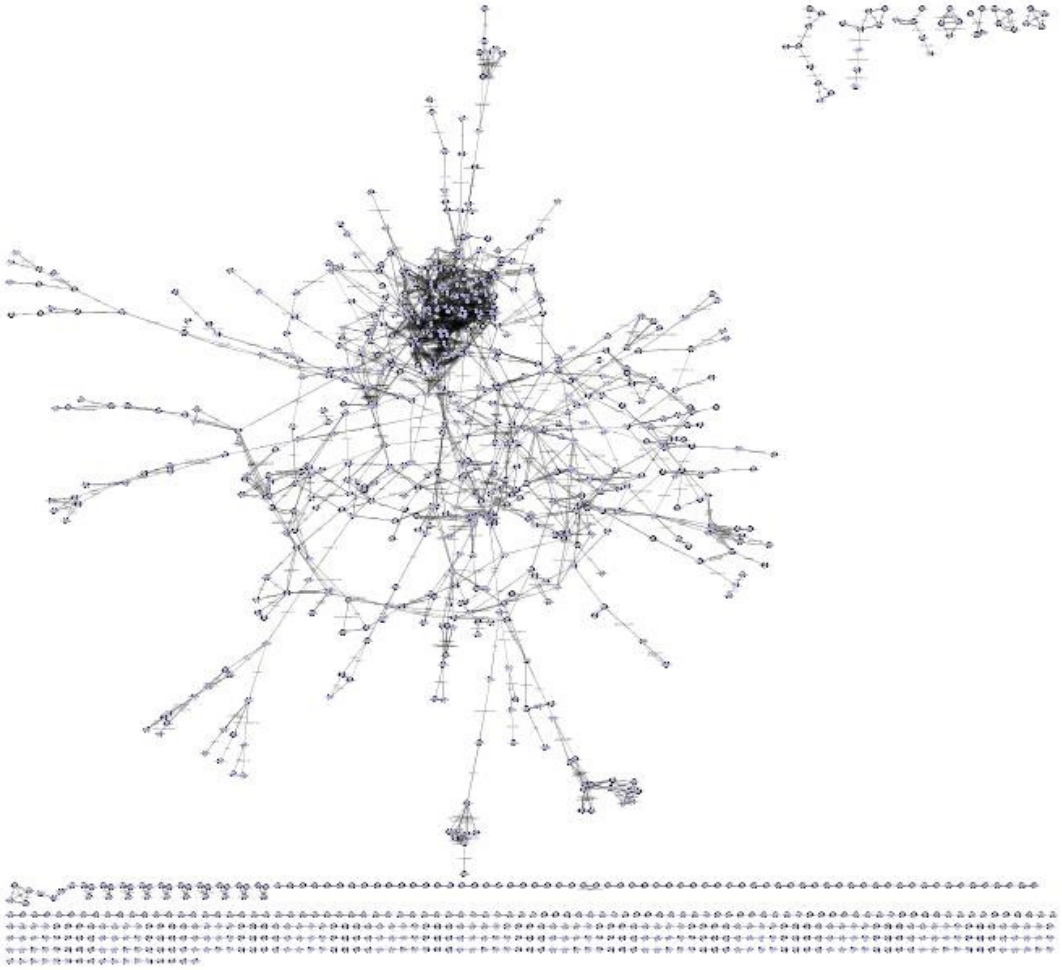


Figure 5-3: CM state network.
Cytoscape representation of extracted metabolites and their relevant *ab initio* biochemical interactions. Metabolites that show no interactions with any other metabolites are shown as individual "orphans" at the bottom.

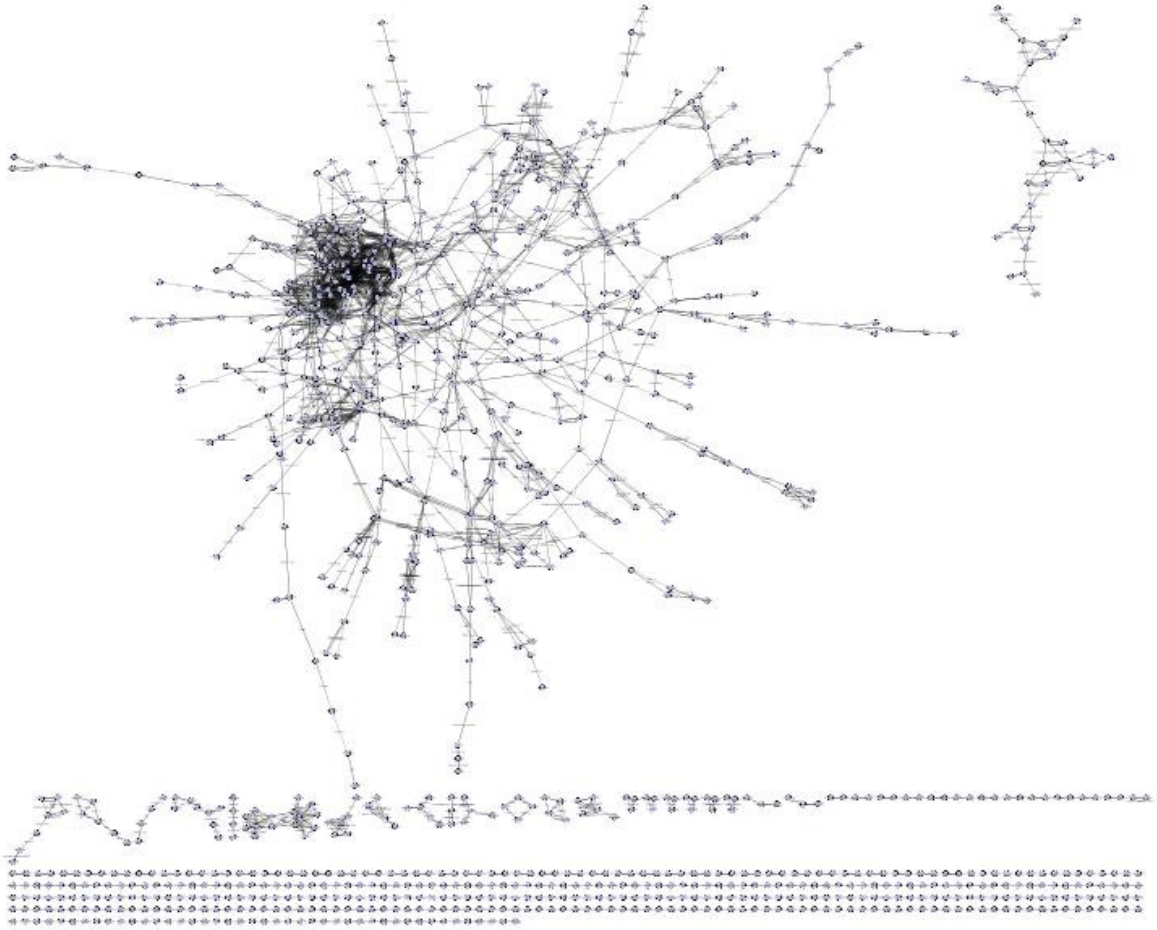


Figure 5-4: GlcNAc state network.
Cytoscape representation of extracted metabolites and their relevant *ab initio* biochemical interactions. Metabolites that show no interactions with any other metabolites are shown as individual "orphans" at the bottom.

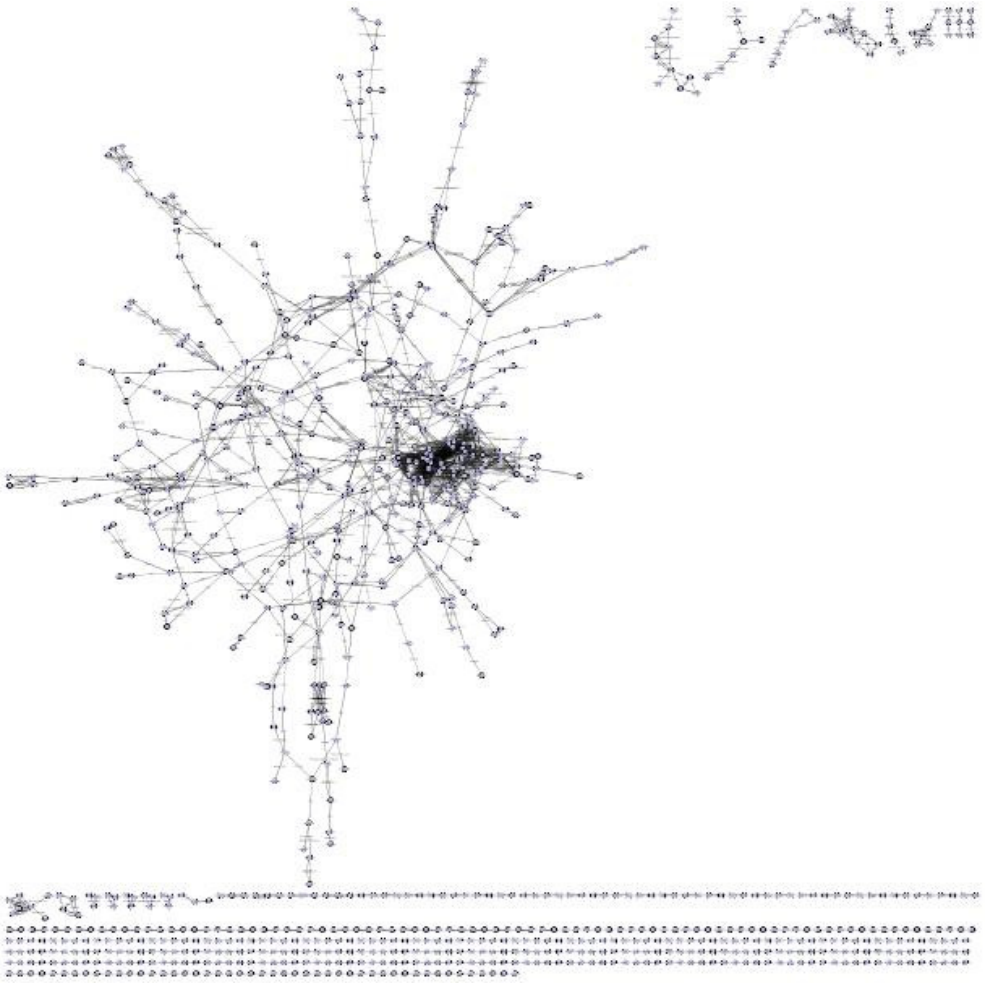


Figure 5-5: Glucose state network.
Cytoscape representation of extracted metabolites and their relevant *ab initio* biochemical interactions. Metabolites that show no interactions with any other metabolites are shown as individual "orphans" at the bottom.

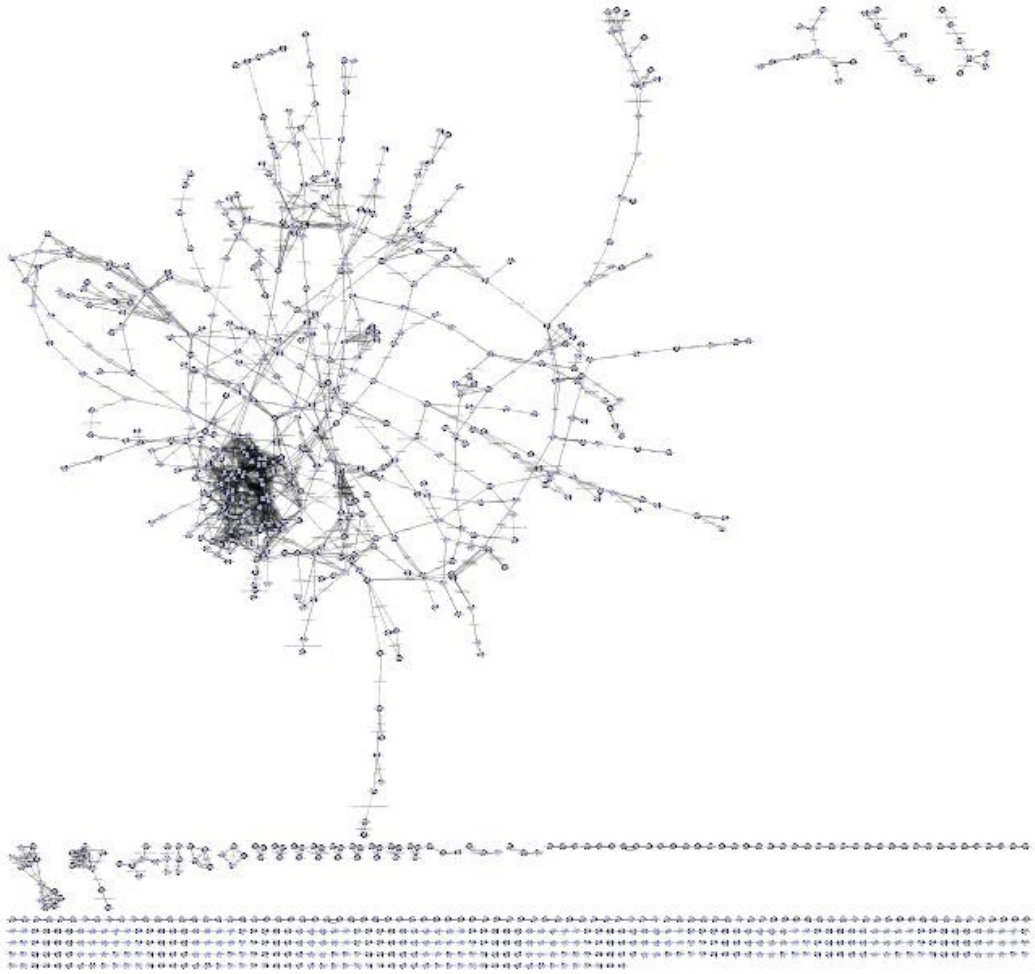


Figure 5-6: Proline state network.
Cytoscape representation of extracted metabolites and their relevant *ab initio* biochemical interactions. Metabolites that show no interactions with any other metabolites are shown as individual "orphans" at the bottom.

In comparing metabolic states (Table 5), the CM state showed 1085 nodes (metabolites) and 1711 edges (interactions) whereas the GlcNAc showed 1113 nodes and 1794 edges. The GL state showed 1011 nodes and 1386 edges whereas the PR state showed 1021 nodes and 1389 edges. In general, the global view of the metabolome constructed by Cytoscape has all linking metabolites forming a network and orphan metabolites (ones that show no interaction with any others) displayed individually.

	Nodes	Edges
CM	1085	1711
NA	1113	1794
GL	1011	1386
PR	1021	1389

Table 5: Total nodes and edges across the metabolic states

Primary investigations took a metabolomic fingerprinting approach. An unbiased global network, built using filtered masses from the Orbitrap data set was then subjected to principal component analysis (M Kamleh, University of Strathclyde). The principal component analysis (PCA) allows representation of complex data in lower dimensional space, defined by the principal components (PCs) (Stoyanova & Brown, 2001). The focus of PCA is pattern recognition, identifying directions of large variations in the data via the principal components (Stoyanova & Brown, 2001). In the case of spectral data, the principal component is amplitude regardless of a lineshape, whereas the other PCs are indications of what other kind of variations are in the data.

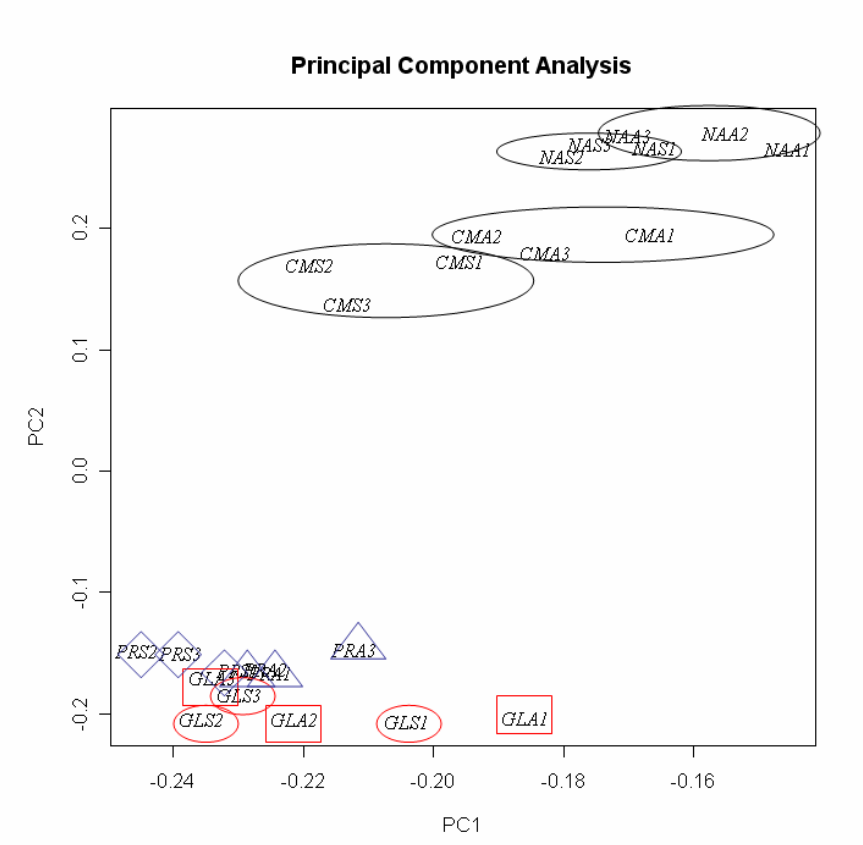


Figure 5-7: Principal Component Analysis.
Principal component analysis scores of the filtered dataset. Cunningham's medium grown samples (CM), Cunningham's medium supplemented with GlcNAc grown samples (NA), proline only medium grown samples (PR), and glucose only medium grown samples (GL). Metabolites extracted from cell pellet (A) or supernatant (S).

The thousands of discrete data points obtained from each sample examined represents the action of many known and unknown variables within the system (Kemsley *et al.*, 2007). The PCA generated is a score plot, with the score a presumed value expressing how close one sample is to the others. Samples behaving in the same way will exhibit close scores.

PCA showed distinct grouping of metabolites derived from cells grown in specific medium, with CM and NA samples (CM based) grouping together and PR and GL samples (SDM80 based) grouping together. It was hypothesised that NA samples should mimic the PR state, considering addition of GlcNAc causes a switch to proline metabolism (chapter 4). However, when viewing the global metabolome, many more factors are at play. And considering the subtle switches in metabolic adaptation which are evident (chapter 4), the full metabolite profile will never be a complete mimic of PR state. Moreover, differences in medium composition play a part, as they determine the resulting metabolic profile. Cunningham's medium is much richer in component mix, exhibiting many more individual components, whereas SDM80 reflects a more restrictive and basic medium composition (Appendix 8.2). The greater the number of starting metabolites available mean the more metabolites as a result of metabolism we are likely to see. Indeed, when viewing the metabolome, more metabolites are seen within the CM network as compared to the network based upon SDM80 medium (GL and PR). With this in mind, the only two conditions which are directly comparable are the GL and PR growth states. The metabolites seen in these two states will reflect a much truer picture of the adaptational processes at play.

5.2.2 Metabolic Fingerprint

The next step was a direct comparison of the metabolites found in the CM state with the NA state as well as between GL and PR states. Comparing the PR and GL states should reveal differences in metabolites as a result of the usage of differing carbon sources. The metabolites that appear as a result of PR usage as compared with GL usage may provide insight into global metabolic state. Moreover, the appearance of metabolites seen in the GlcNAc state as compared with the CM state may reveal metabolites linked to the metabolic shift as seen in comparing the GL and PR state. Also, it is important to focus on appearing metabolites that form networks as that will highlight new pathways and patterns.

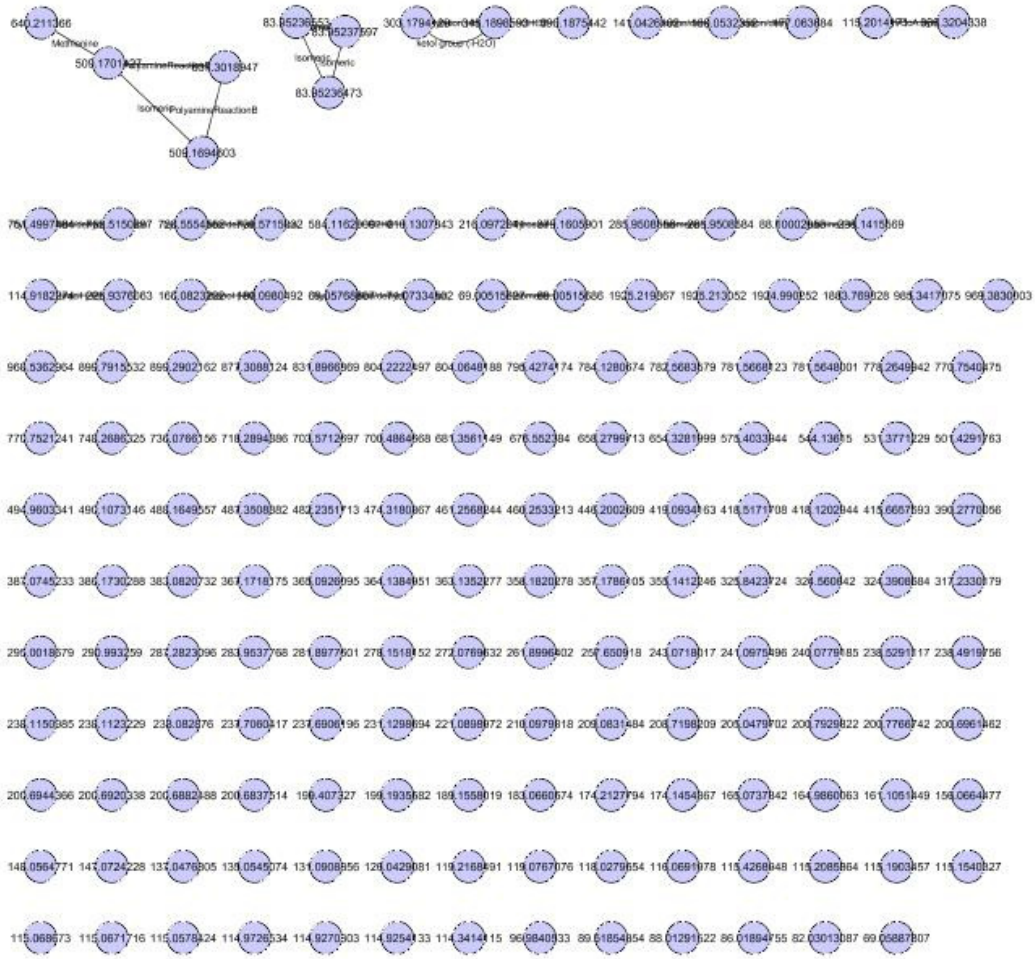


Figure 5-8: Metabolites unique to proline metabolism (comparing GL and PR states). Cytoscape representation of extracted metabolites and their relevant *ab initio* biochemical interactions. Metabolites that show no interactions with any other metabolites are shown as individual "orphans" at the bottom.

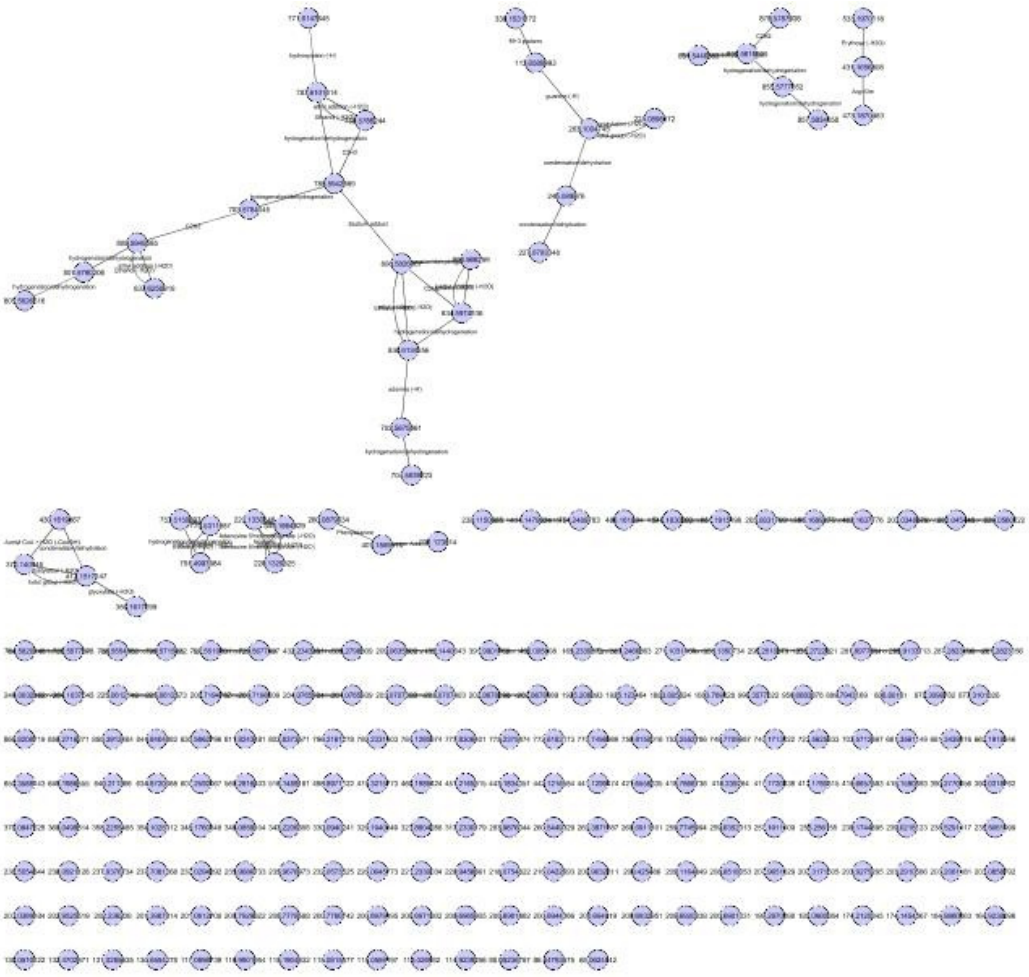


Figure 5-9: Metabolites unique to GlcNAc supplementation (comparing CM and NA states). Cytoscape representation of extracted metabolites and their relevant *ab initio* biochemical interactions. Metabolites that show no interactions with any other metabolites are shown as individual "orphans" at the bottom.

Identified from the PR state were 166 metabolites unique to proline metabolism (Figure 5-8). In comparison, GlcNAc supplementation of CM showed 277 metabolites unique to that particular growth condition (Figure 5-9). Of the 277 metabolites that appear as a result of GlcNAc supplementation, 28 (~10%) were common to PR state (Table 6), suggesting that these metabolites are associated with proline metabolism.

Conversely, we wanted to apply the same characterisation to the GL and CM state respectively in comparison to the PR and NA state, showing metabolites unique to glucose metabolism.

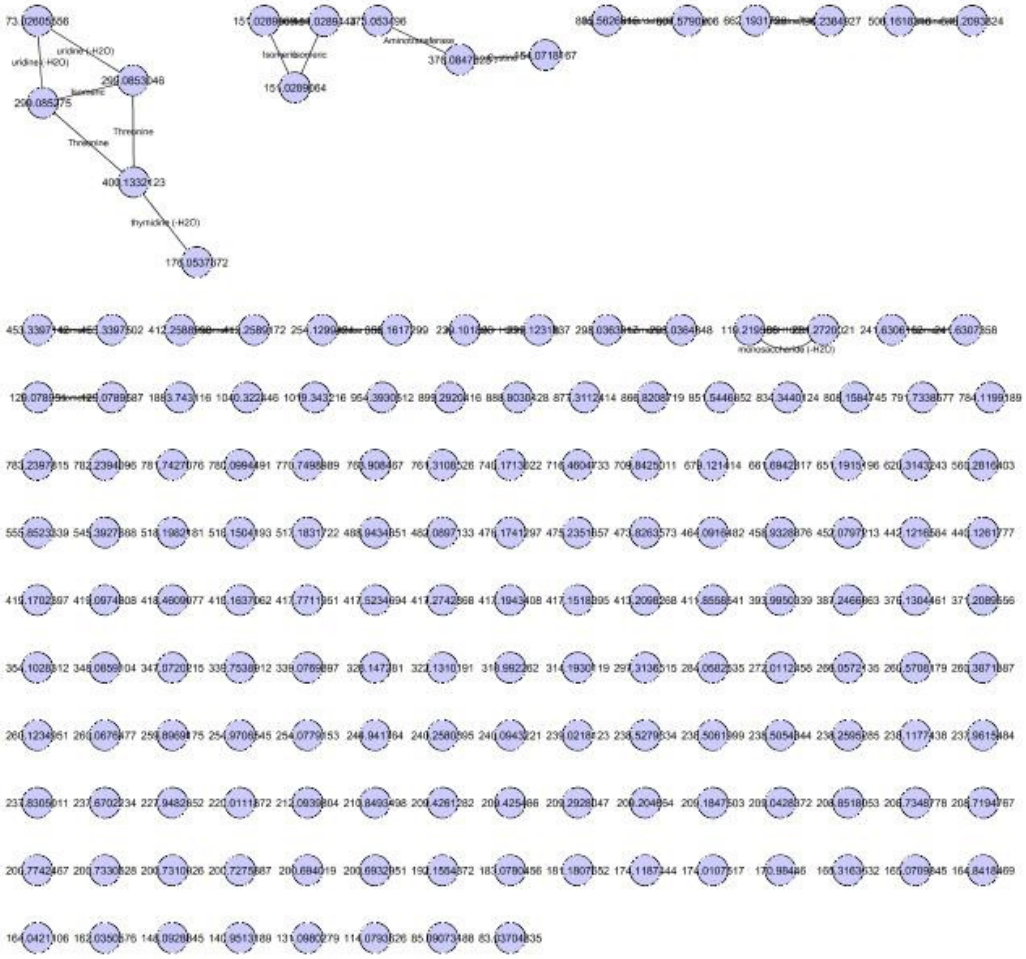


Figure 5-10: Glucose specific metabolites (comparing PR and GL states).
Cytoscape representation of extracted metabolites and their relevant *ab initio* biochemical interactions. Metabolites that show no interactions with any other metabolites are shown as individual "orphans" at the bottom.

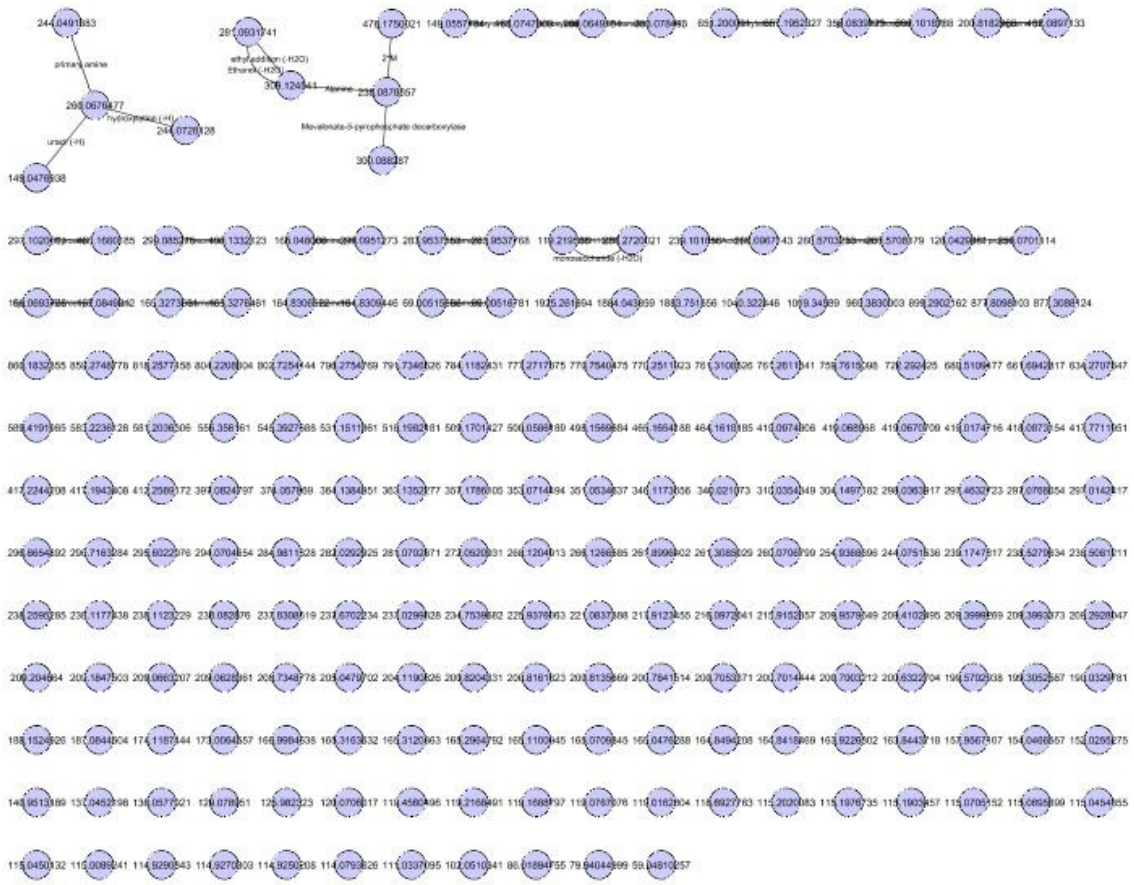


Figure 5-11: Glucose specific metabolites (comparing NA and CM states).
Cytoscape representation of extracted metabolites and their relevant *ab initio* biochemical interactions. Metabolites that show no interactions with any other metabolites are shown as individual "orphans" at the bottom.

Identified from the GL state were 159 metabolites unique to glucose metabolism (Figure 6-10). In comparison, the CM state showed 207 metabolites unique to that particular growth condition (Figure 6-11). Of the 159 metabolites associated with glucose metabolism, 37 (~23%) were common to the CM state (Table 6).

metabolites unique to proline metabolism			metabolites unique to glucose metabolism			
115.190	221.089	681.356	114.079	237.670	298.036	545.392
174.145	238.115	703.571	119.219	237.830	299.085	661.694
174.212	238.529	728.555	140.951	238.117	299.085	761.310
180.098	281.897	730.571	165.071	238.259	400.133	899.292
200.692	287.282	751.499	165.316	238.528	412.258	1040.322
200.694	317.233	753.515	174.118	239.101	412.258	
200.696	390.277	784.128	208.734	260.067	417.194	
200.776	415.665	1883.769	209.184	260.570	417.771	
200.793	488.165		209.204	281.272	419.097	
208.719	640.211		209.292	298.036	482.089	

Table 6: Carbon source specific metabolites (pellet).

The generated list of metabolites specific for glucose and proline associated metabolism represents, essentially, a list of biomarkers for metabolism. The exact masses derived from the pellet are assigned putative formulae using an algorithm that sums all possible combinations of the elements C, H, O, P, N, S, Cl, allowing possible putative compounds to be postulated.

Pellet			
Proline State		Glucose State	
Mass	Formula	Mass	Formula
174.212	C5H14O4N3	114.079	C5 H10 O1 N2
221.089	C7H9O1N8	165.070	C5H15N2P2
238.115	C6H16O5N5	238.117	C9H14O2N6
281.897	C7H4O4CL1P1S2	239.101	C9H13O3N5
287.282	C17H37O2N1	260.067	C4H16O4N5P2
317.233	C17H33O5	281.272	C18H35O3N5
390.277	C24H38O4	298.036	C7H14O6N3P2
488.164	C24H35O4CL2P1	299.085	C9H17O10N1
640.211	C27H38O4N8CL2S1	299.085	C9H17O10N1
681.356	C29H53O14N4	400.133	C19H22N6CL1P1
		412.258	C18H35O2N7P1
		412.258	C18H35O2N7P1
		417.194	C22H30O4N2P1
		419.097	C7H20O7N10P1S1
		482.089	C21H18O3N7CL1P1
		545.392	C29H55O8N1
		761.310	C29H48O13N9P1
		899.292	C28H57O15N10P2S2

Table 7: Putative formula generated from pellet metabolites.

Not all metabolites could be assigned putative formula within an appropriate range of error (2 ppm). The range of error (in parts per million) refers to how far the detected mass deviates from the standard predicted mass. The metabolites that could be assigned putative formula could not be identified by PubChem. However, the biomarkers generated here can provide a phenotype for metabolic adaptation.

5.2.3 Metabolic Footprint

Metabolomic footprinting involves the analysis of extra-cellular metabolites in cell culture medium, which is a reflection of metabolites taken up or excreted by cellular processes. We further wanted to characterise each metabolic state by identifying metabolites that appear as a result of differing carbon source usage. A total of 175 metabolites were found to be unique to the PR state and 123 to the GL state (in comparison to the GL state and PR state, respectively). Whereas 223 metabolites were found to be unique to the NA state and 191 to the CM state (in comparison to the CM state and NA state, respectively).

metabolites unique to proline metabolism			metabolites unique to glucose metabolism	
73.026056	174.2125	417.1982	105.0426	650.3568
83.037048	200.6972	419.0934	120.0706	878.3125
83.952363	200.7767	473.8264	149.0477	910.2836
114.92541	200.7824	498.157	151.0289	
115.00889	208.8514	589.4192	165.234	
115.20142	237.9377	748.2686	208.9397	
115.42682	238.082	782.2394	417.8282	
117.06744	238.1123	877.813	418.461	
150.05031	260.096		431.273	
165.07378	390.277		473.187	

Table 8: Carbon source specific metabolites (supernatant).

This identified, as a product of glucose metabolism, 13 unique metabolites. Conversely, proline metabolism showed 28 unique metabolites. Of all the metabolites identified only one could be assigned chemical formula. The glucose-specific metabolite of mass 105.042 corresponds to a chemical formula of C3H7NO3 (Serine). It is possible that there is a greater need for serine when proline is being used as the main carbon source, leading to a greater consumption of the metabolite from the surrounding medium.

The exact masses derived are assigned putative formulae using an algorithm that sums all possible combinations of the elements C, H, O, P, N, S, Cl, allowing possible putative compounds to be postulated.

Supernatant			
Proline		Glucose	
Mass	Formula	Mass	Formula
83.037	C4H5O1N1	208.939	C2H1O5N3P2
150.050	C9H10S1	431.273	C17H35O5N8
165.073	C2H9O3N6	473.187	C17H27O9N7
238.112	C13H19O2P1	650.356	C27H54O7N7P2
260.095	C17H12O1N2		
390.277	C24H38O4		
417.198	C27H30O2P1		
498.156	C21H30O7N3P2		
589.419	C30H53O4N8		
748.268	C29H45O15N6P1		

Table 9: Putative formula generated from supernatant metabolites.

Not all metabolites could be assigned putative formula within an appropriate range of error (2 ppm). The metabolites that could be assigned putative formula could not be identified by PubChem. However, the biomarkers generated here can provide a phenotype for metabolic adaptation.

5.2.4 Metabolic Profile

Metabolites of the glycolytic pathway and key mitochondrial metabolites were searched for. This was relevant since previous work (chapter 4) had indicated differences in energy metabolism related to these pathways. It was important; first of all, to find out if we could view all the relevant metabolites (glycolysis and Kreb's cycle intermediates) under the conditions used (extraction of metabolites and Orbitrap processing technique). The molecules with the same mass but distinct structures (dihydroxyacetone phosphate and glyceraldehyde-3-phosphate) can only be discriminated with additional information, and is beyond the scope of what is investigated here. Of all the glycolytic and mitochondrial intermediate metabolites only pyruvate (Figure 5-12) and the amino acids (proline, threonine, glutamine and glutamate) (Figure 5-13) could be identified within the dataset.

Pyruvate is the ultimate end product when glucose is being used as the primary carbon source (Figure 1-4). However, when proline is being used, pyruvate is further metabolised within the mitochondrion. We would therefore expect to see a greater abundance of pyruvate in GL and CM states as compared to NA and PR states.

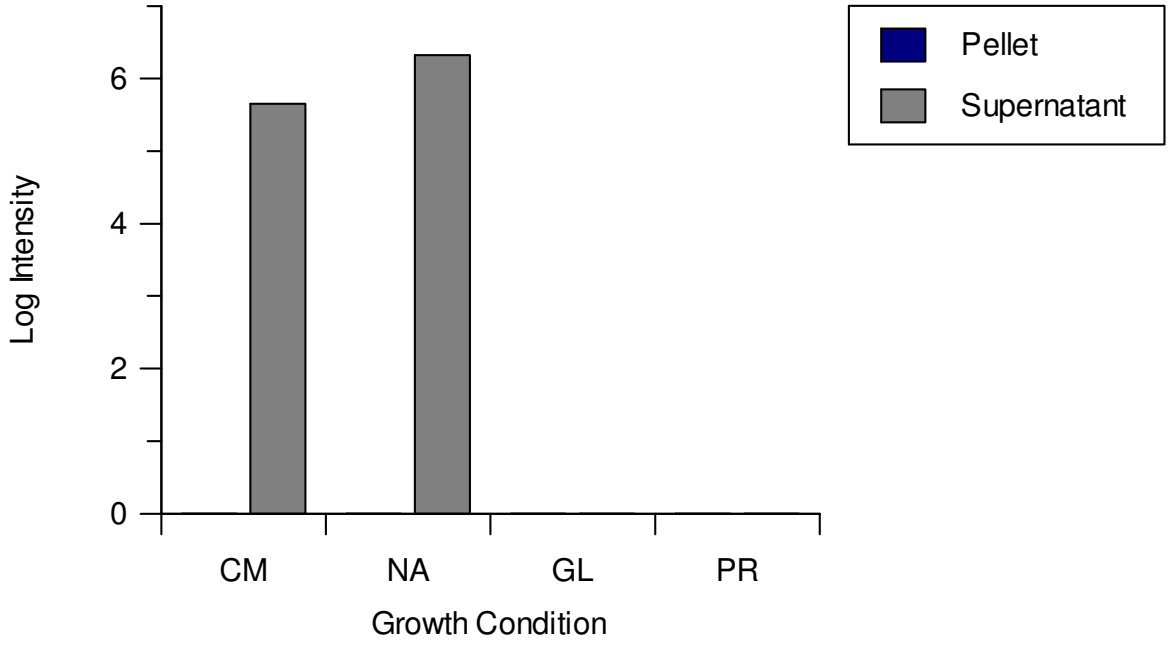


Figure 5-12: Pyruvate abundance within the cell pellet and supernatant.
Relative intensity of pyruvate found in the cell pellet and supernatant from cells grown in differing conditions; Cunninghm's medium (CM), CM supplemented with GlcNAc (NA), SDM80 plus Glucose (GL), SDM80 plus Proline (PR).

Within the cell pellet no pyruvate was identified, where as high amounts of pyruvate could be seen in the supernatant extract of the CM and NA conditions. SDM80 contains twice as much pyruvate as Cunningham's medium (100 mg/L vs 200 mg/L; Appendix 8.2, 8.4) Here, comparing pyruvate abundances across the four states does not give any insight into the metabolic status of the cell.

Continuing the metabolic profiling approach we wanted to investigate other branches of the metabolome. More specifically, the amino acid complement (as analysed by targeted metabolite analysis) of PCF trypanosomes with regard to the metabolic adaptation as a result of differing carbon source usage. Amino acid usage regulation has already been seen with proline (chapter 4). However it is not known if glucose exerts effects (whether repressive or not) on other amino acids, or if other amino acids become more essential in the absence of glucose. The full conventional 20 amino acid complement along with related compounds (taurine, ornithine homocysteine, and citrulline) was sought within the data generated from the Orbitrap process (Table 10).

	Pellet				**Supernatant**			
	CM	NA	GL	PR	CM	NA	GL	PR
Glycine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Alanine	7.434	6.641	7.384	7.390	6.573	7.186	7.321	7.311
Serine	5.832	5.609	5.923	5.551	5.434	0.000	5.519	0.000
Proline	8.627	8.647	7.761	8.332	8.630	8.658	7.311	8.319
Threonine	7.063	7.132	7.399	7.439	7.063	6.950	7.345	7.364
Cysteine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Taurine	7.290	7.319	7.496	7.466	7.303	7.319	7.549	7.507
Leucine	8.062	8.072	8.085	8.096	8.057	8.063	8.044	8.050
Asparagine	6.439	6.996	5.556	5.628	6.512	6.869	0.000	0.000
Ornithine	7.378	7.430	7.197	7.142	7.415	7.449	7.186	7.121
Aspartic acid	6.230	6.531	0.000	0.000	5.863	6.242	0.000	0.000
Homocysteine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Glutamine	7.218	7.182	6.090	6.077	7.231	7.374	6.023	6.769
Lysine	7.701	7.687	7.615	7.658	7.727	7.700	7.578	7.609
Glutamate	6.991	7.060	6.660	6.806	6.981	7.046	6.650	6.681
Methionine	7.940	7.985	8.021	8.039	7.967	7.991	8.020	8.012
Histidine	8.115	8.105	7.907	7.976	8.134	8.116	7.886	7.926
Phenylalanine	8.558	8.395	8.541	8.584	8.585	8.389	8.543	8.546
Arginine	8.680	8.649	8.650	8.712	8.548	8.655	8.633	8.509
Citrulline	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Tryptophan	7.214	7.305	6.679	6.673	7.210	7.314	6.618	6.626
Tyrosine	8.043	8.054	8.205	8.143	8.083	8.075	8.219	8.159
Valine	7.557	7.578	7.689	7.604	7.415	7.561	7.644	7.552
Isoleucine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table 10: Targeted amino acid analysis.

Relative intensities of amino acids found in cell pellet and supernatant of cells grown in differing conditions; Cunningham's medium (CM), CM supplemented with GlcNAc (NA), SDM80 plus Glucose (GL), SDM80 plus Proline (PR).

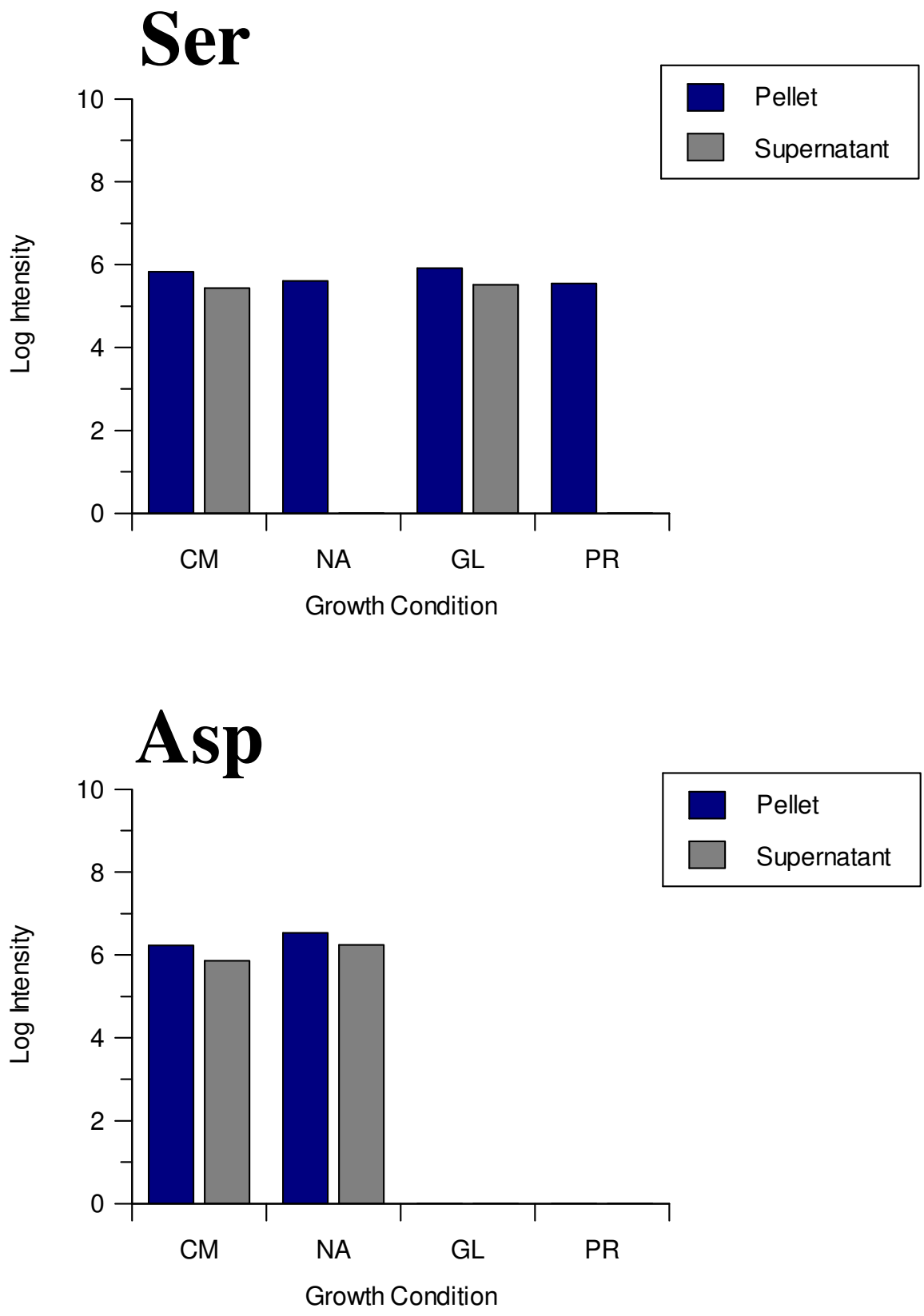


Figure 5-13: Targeted amino acid analysis.
Relative intensities of serine (top) and aspartate (bottom) as found in the cell pellet and supernatant of cells grown in differing conditions.

Of the 20 amino acids only glycine, cysteine and isoleucine could not be identified from the list of masses, and only serine and aspartate showed any significant differences (Figure 5-13). Most amino acids showed no considerable changes across the differing growth conditions. Within the cell pellet no aspartate was found in either the GL or PR states in comparison to the CM and NA states (despite aspartate being a medium component in both). This was also mimicked in the profile seen from the supernatant. This could possibly be explained by the fact that CM contains 110 mg/L of aspartate whereas SDM80 contains only 13.3 mg/L, and the fact we do not see any aspartate in the GL and PR states is because the little aspartate that is present is quickly metabolised.

Interestingly, serine was present only in the supernatant of the CM and GL states (absent in the NA and PR states). This variance was not mimicked in the profile seen from the cell pellet. It is possible that there is a greater need for serine when proline is being used as the main carbon source, leading to a greater consumption of the metabolite from the surrounding medium. The fact that a greater amount of serine within the cell pellet was not seen does not refute that possibility as an increase in serine metabolism could remove the metabolite from the profile seen, leaving us with an interior metabolite abundance similar to the GL state.

5.3 Discussion

Systems biology offers an attractive method of investigating cellular processes at a global level. The advantage of global metabolomic approaches is that it highlights various cellular processes that can be investigated further by classical biochemical targeted techniques. Indeed, that was our intention here. We aimed to use metabolomic approaches not only to visualise the metabolome of the trypanosome as a result of differing carbon source usage, but also identify biomarkers that appear as a result of the metabolic adaptations already characterised (Lamour *et al.*, 2005; chapter 4).

Metabolomics technologies are embryonic. Refinements are required, not only to the sample processing techniques (to allow more metabolites to be seen) but also to the data analysis. This study represents one of very few performed on any organism and it is the eventual goal to develop methodologies adapted to the investigation of the *T. brucei* metabolome. This preliminary data highlights areas that need further confirmation in terms of reproducibility and areas of methodology to troubleshoot. Whether metabolomics will prove an effective tool for the characterisation and analysis of cellular processes remains to be seen.

Our initial assumption was that the metabolic adaptations involved in differing carbon source usage would be an easy event to observe in trypanosomes when viewing the global metabolome. There is a switch from the glycosome to the mitochondrion as the major source of energy production that occurs with the metabolic adaptation. It was our assumption that we would observe more intermediates specific for each pathway depending on the carbon source in use. This was not the case. For example, proline was seen in equal amounts irrespective of primary carbon source. The same was true for glutamate, a key intermediate in proline metabolism.

What global metabolomics offers is a snapshot of the processes ongoing. It has the disadvantage of not being able to show flux through a specific pathway. Varying and having more incubation time points might allow for metabolic flux to be seen. It is possible that the metabolites not seen from the data could be simply because they have been used up quickly. Moreover, metabolites not seen can be as a result the extraction process. The use of more than one extraction process will undoubtedly identify metabolites not common to another extraction process. For example, extraction of cells into boiling water is a method commoly employed as complementary to boiling ethanol. A greater portion of phospholipids might be precipitated in EtOH extraction as compared with water extraction (M Kamleh, personal communication). The methodology employed consists of a centrifugation step that separates cells from spent medium. This centrifugation step can be eliminated as it perturbs cellular metabolism by creating an anoxic (depleted oxygen) environment. Immediate addition of boiling EtOH will give a much truer snapshot of the metabolome.

The orbitrap process can also be altered to allow more metabolites to be seen. During the process, metabolites are ionised by the addition of a proton. It is also possible to run the process in reverse by stripping away a proton. This means that metabolites that do not favour proton addition might be more amenable to subtraction (sugar phosphate compounds), allowing them to be identified (M Kamleh, personal communication). With all these improvements to the methodology in mind, it is still possible that targeted metabolic profiling will support the hypothesis already confirmed by traditional biochemical techniques.

Targeted amino acid analysis highlighted serine as a metabolite that differs as a result of metabolic adaptation. Serine was absent from supernatant extracts in both the PR and NA state, suggesting that when cells are using proline as the principal carbon source there is a

greater metabolic requirement. This might translate into upregulation of transporter processes (e.g. TbAATP1; chapter 3) and/or target enzyme relevant to serine metabolism.

Metabolic fingerprinting involves an unbiased global screening approach. The intention is not to identify each observed metabolite but allows comparison of patterns or "fingerprints" as a result of cellular perturbations. Fingerprinting observes the true phenotype, however, the causal metabolites of the observed phenotype must be identified if any biochemical understanding is to be gained from the data. Identified from metabolomic fingerprinting investigation was essentially a list of metbolites unique to either L-proline or D-glucose metabolism. Of the 159 metabolites associated with glucose metabolism (SDM80 plus glucose), 37 (~23%) were found in the CM state; whereas of the 277 metabolites that appear as a result of GlcNAc supplementation, 28 (~10%) were common to PR state. These handful of metabolites represent biomarkers for metabolism. However, these biomarkers could not be assigned putative chemical formula, and hence, the metabolite could not be identified. This is possibly due to the fact that metabolites appear in a range of different forms. Some metabolites appear associated with sodium ions. Many others forms of adducts exist, K adduct, Ca adduct, CH3CN adduct, NH4 adduct, HCOOH adduct, clusters of 2 or more molecules, as well as triple and quadrable charged species. At present these cannot be easily be deciphered. What needs to be done is to extend the simple putative formula analysis to include all other permutations and combinations that might exist for any given mass.

There is no doubt that metabolomics offers new areas of trypanosome biology to explore. With only half the genome annotated there is potential for new biochemical pathways to be identified, whether they are trypanosome-specific or not. Further investigations need to confirm these biomarkers as well as confirm if these metabolites appear as a result of other conditions that cause a metabolic shift.

6 Uptake and Effects of Trypanotoxic Amino Acid Analogues

6.1 Introduction

The precedent set by work with the P2 transporter, with regards to targeting toxins to trypanosomes via nutrient transporters (chapter 1.11), inspired a search for several amino acid analogues with trypanocidal activity. Some amino acid transporters fulfil similar criteria to purine transporters with regard to their transport characteristics (Hasne & Barrett, 2000). In some cases, the transporters' natural substrates are of low plasma abundance, so these transporters should have correspondingly high affinities for their substrate (and hence maybe also substrate analogues). A drug using such a carrier will also have relatively low competition from natural substrate. Several amino acid analogues that kill trypanosomes are known. Some thiazine-linked amino acids showed good *in vitro* activity against *T. brucei* and also led to temporary reduction of parasitaemia in mice (Vanbogaert *et al.*, 1993). These compounds had been designed as putative inhibitors of trypanothione biosynthesis, although it appears that this was not their mode of action, which remains uncertain. Most recently, Acivicin and 6-diazo-5-oxo-l-norleucine (DON); which are glutamine analogues that can kill trypanosomes *in vitro* (Hofer *et al.*, 2001). These glutamine analogues only arrest the proliferation of the parasites. A functional immune response is then required to eradicate the static trypanosomes (Fijolek *et al.*, 2007). Also, the methionine analogue buthionine sulphoxamine (BSO) has been identified as potential trypanocides (Huynh TT *et al.*, 2003; Arrick *et al.*, 1981) giving a 50 µM IC_{50} against PCF trypanosomes. BSO mode of action seems to involve inhibition of γ-Glutamylcysteine synthetase (γ-GCS) resulting in trypanolytic effects. However, γ-GCS inhibition was not conclusively shown to be the sole target for BSO.

6.1.1 Azaserine

Compounds affecting the utilisation of the amino acid L-glutamine have a comparatively long history in the field of cancer chemotherapy (Livingston *et al.*, 1970). At one time the glutamine agonists were undergoing clinical trials, but lack of effectiveness has caused a decline in interest. Glutamine, in trypanosomes, is essential for several biosynthetic pathways as well as DNA replication. Azaserine is of similar structure to glutamine and is an irreversible inhibitor of glutamine amidotransferases (Livingston *et al.*, 1970), which catalyze the ATP-dependent transfer of the amido nitrogen of glutamine to an acceptor – in this case GMP (Figure 6-1).

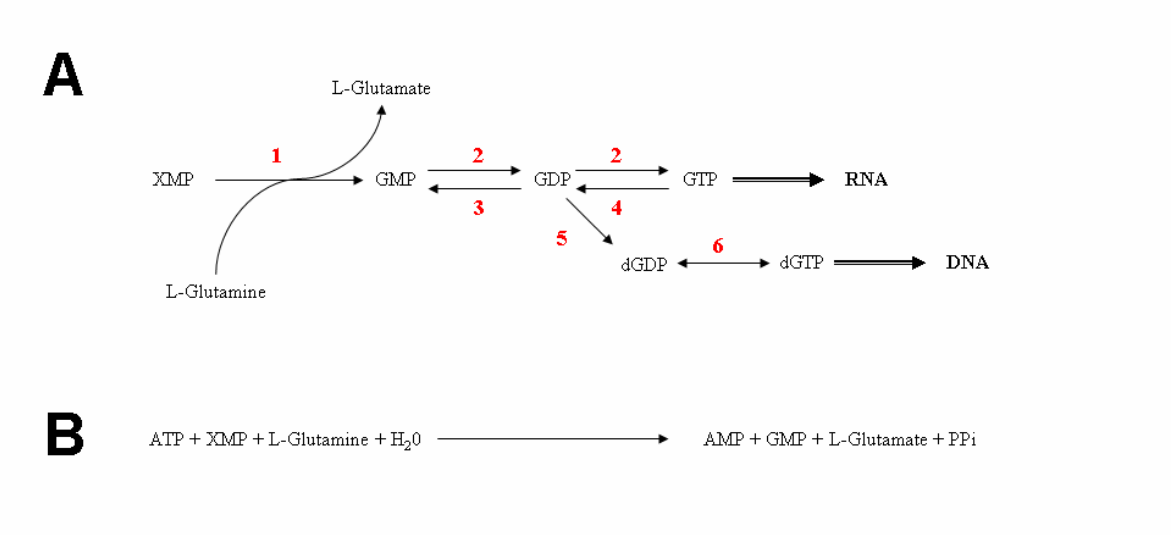


Figure 6-1: Schematic representation of DNA synthesis showing L-glutamine usage in *T. brucei* (A) and the specific chemical reaction (B).
Enzymatic steps are represented by arrows with enzymes represented by red numbers (1, GMP synthase; 2, guanylate kinase; 3, nucleoside diphosphatase; 4, pyruvate kinase; 5, ribonucleotide-diphosphate reductase large chain; 6, nucleoside diphosphate kinase) Abbreviations: XMP, xanthosine monophosphate; GMP, guanosine monophosphate; GDP, guanosine diphosphate; GTP, guanosine triphosphate; dGDP, deoxyguanosine diphosphate; dGTP, deoxyguanosine triphosphate; PPi, diphosphate.

Azaserine is the most thoroughly studied compound within the diazo-analogues of glutamine. In mammalian cells, it has been shown that azaserine interferes with several reactions that involve glutamine. The reaction most sensitive to inhibition by azaserine is the conversion of formyglycinamide ribotide (FGAR) to formylglycinamidine ribotide (FGAM), a reaction within the biosynthetic pathway of purines (Hartman *et al.*, 1955). The mechanism of inhibition still remains unclear, but what is clear is the irreversibility of the azaserine effect. *De novo* inhibition of purine biosynthesis is not the only cellular affect of azaserine. There has also been evidence shown for inhibition of cytidine biosynthesis and binding to DNA to interferes with its template function (Livingston *et al.,* 1970).

Azaserine has been shown to have some antibiotic properties (Bennett *et al.*, 1956). In prokaryotes, azaserine is transported by the aromatic transport system. In *Salmonella typhimurium*, resistance seems to be as a result of a mutation in a gene whose product transports general aromatic amino acids (Williams *et al.*, 1980). Sensitivity is regained by another mutation in a gene involved in histidine transport, suggesting that the antibiotic may be transported by multiple transport systems.

Azaserine action on *Escherichia coli* is similar to that of mammalian cells. The aromatic amino acids are the most effective in blocking the inhibition by azaserine on growth of *E. coli*. However, the ability of the aromatic amino acids to antagonise azaserine action may be due to non-specific effects and is not related to the action of azaserine on the aromatic acid biosynthesis pathway (Kaplan *et al.*, 1959). The results of their investigation suggest that the penetration of the antibiotic through the cell membrane is prevented by the aromatic amino acids. Hypotheses proposed have been supported by the fact that azaserine has been shown to be under the control of the amino acid transport system (Jacquez *et al.*, 1957). However, azaserine action on the utilisation of aromatic amino acids in *E. coli* has not been investigated and cannot be ruled out.

Trypanosomes lack aromatic amino acid biosynthesis pathways as well as most of the enzymes of the classical pathways for aromatic amino acid oxidation. Conversion of phenylalanine to tyrosine by phenylalanine-4-hydroxylase does not occur in *T. brucei*. All trypanosome species have genes for transamination and reduction to the corresponding aromatic lactate derivative (Berriman *et al.*, 2005). This suggests that action of azaserine does not involve aromatic amino acid pathways, leaving competition for glutamine in GMP and CTP synthesis as the key candidate for azaserine's trypanocidal action, as *de novo* purine biosynthesis does not occur in trypanosomes.

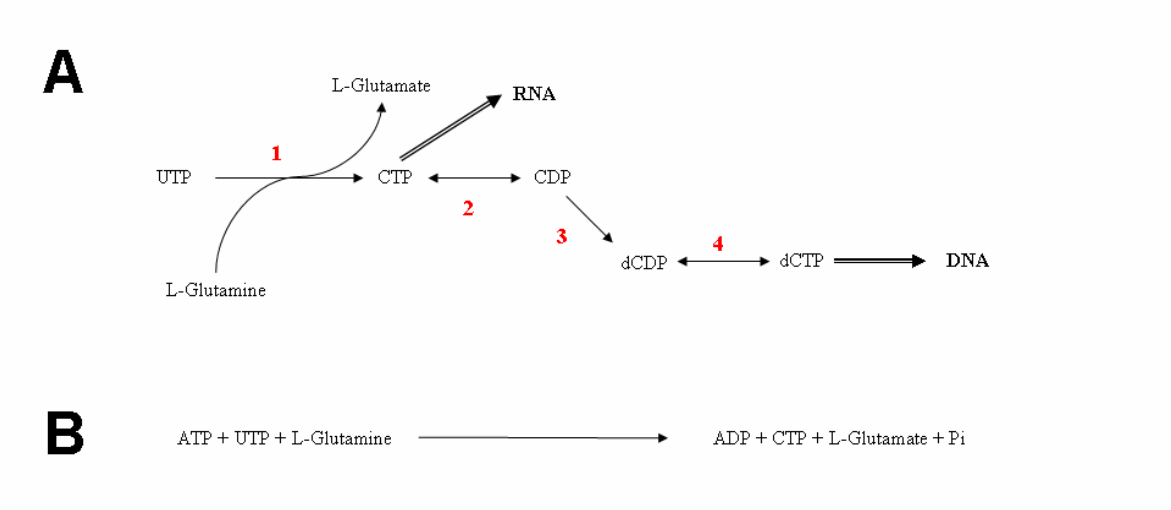


Figure 6-2: Schematic representation of CTP synthesis showing L-glutamine usage in *T. brucei* (A) and the specific chemical reaction (B).
Enzymatic steps are represented by arrows with enzymes represented by red numbers (1, CTP synthase; 2, nucleoside diphosphate kinase; 3, ribonucleoside-diphosphate reductase large chain; 4, nucleoside diphosphate kinase. Abbreviations: UTP, uridine triphosphate; CTP, cytosine triphosphate; CDP, cytosine diphosphate; dCDP, deoxycytosine diphosphate; dCTP, deoxycytosine triphosphate; Pi, inorganic phosphate.

In early studies, azaserine was noted to have an effect on the incorporation of purines and pyrimidines in *Trypanosoma equiperdum* (Momparler & Jaffe, 1964). The amino acids glutamine, tryptophan, tyrosine and leucine partially prevented the inhibition produced by azaserine. Results presented by Momparler & Jaffe (1964) suggest that the effect of azaserine is to inhibit the conversion of UMP to CMP, resulting in a depletion of cytosine-containing nucleotides (Figure 6-2). More recently, CTP synthetase has been shown to be a potential drug target in BSF trypanosomes (Hofer *et al.*, 2001; Fijolek *et al.*, 2007). With CTP levels in BSF trypanosomes controlled by synthesis rather than by degradation, and no apparent salvaging of cytidine or cytosine by the parasite, the inhibition of CTP synthesis has been shown to have a dramatic effect on CTP levels and cell growth.

6.1.2 Levodopa

Potential trypanocidal amino acid analogues may be hampered by size of the side chain to allow targeting via an amino acid transporter. Aromatic amino acid analogues provide a level of complexity that can be built into a molecule and still be targeted through an amino acid transporter. The tyrosine analogue L-Dopa, along with other catecholamines, have been shown to have high trypanocidal activity (Owolabi *et al.*, 1989). Various catecholamines (dopamine and tryptamine analogues) were tested against trypanosomes using a feeder layer culture system (*Microtus agrestis* embryonic fibroblasts). Catecholamines are compounds derived from tyrosine containing the catechol and amine structure. *In vitro* tests showed as little as 1 μM cleared all trypanosomes at a starting density of 10^5 cells per ml after 24 hours. L-Dopa and other catecholamines are subject to auto-oxidation and are potent producers of hydrogen peroxides, quinones, and free radicals.

The discovery of L-Dopa's trypanocidal activity together with the fact that certain catecholamines have been shown to have high trypanocidal activity (Owolabi *et al.*, 1989) lead the investigation into the possibility of metabolic activation. Two pathways exist in mammalian cells for the metabolism of aromatic amino acids. The tyrosine pathway converts tyrosine to noradrenalin via L-Dopa and dopamine (Figure 6-3), whereas tryptophan is converted to melatonin via serotonin (Figure 6-4).

L-Dopa can cross the blood-brain barrier and is commonly used in the treatment of Parkinson's disease (PD). PD is a chronic disease of the central nervous system caused by lowered levels of the inhibitory neurotransmitter dopamine. Symptoms include muscular tremors and weakness. First described over 200 years ago, Parkinson's disease is one of the

most common human degenerative disorders (Schapira, 2005). L-Dopa is the drug most commonly used to address the dopaminergic features of the disease, leaving the long term progression unaffected. The use of dopaminergic drugs improves motor function, and significantly reduces both the morbidity and mortality in sufferers – leading to an overall improvement in quality of life. Progression of the disease is still ongoing, and because of that, the effects of L-Dopa are modified due to the fact that the dopaminergic cells required to store and release dopamine are still in decline.

Two different types of dopamine receptors are found in the brain (D_1-like and D_2-like receptors) and mediate different actions when stimulated by dopamine. Both are G-protein-coupled, D_1-like receptors stimulate adenylyl cyclase whereas D_2-like receptors inhibit adenylyl cyclase (Mercuri & Bernardi, 2005). Despite the fact that D_2-like receptor agonists are more effective anti-parkinsonian drugs, the use of mixed D_1 and D_2 receptor agonists provide the best control over PD symptoms.

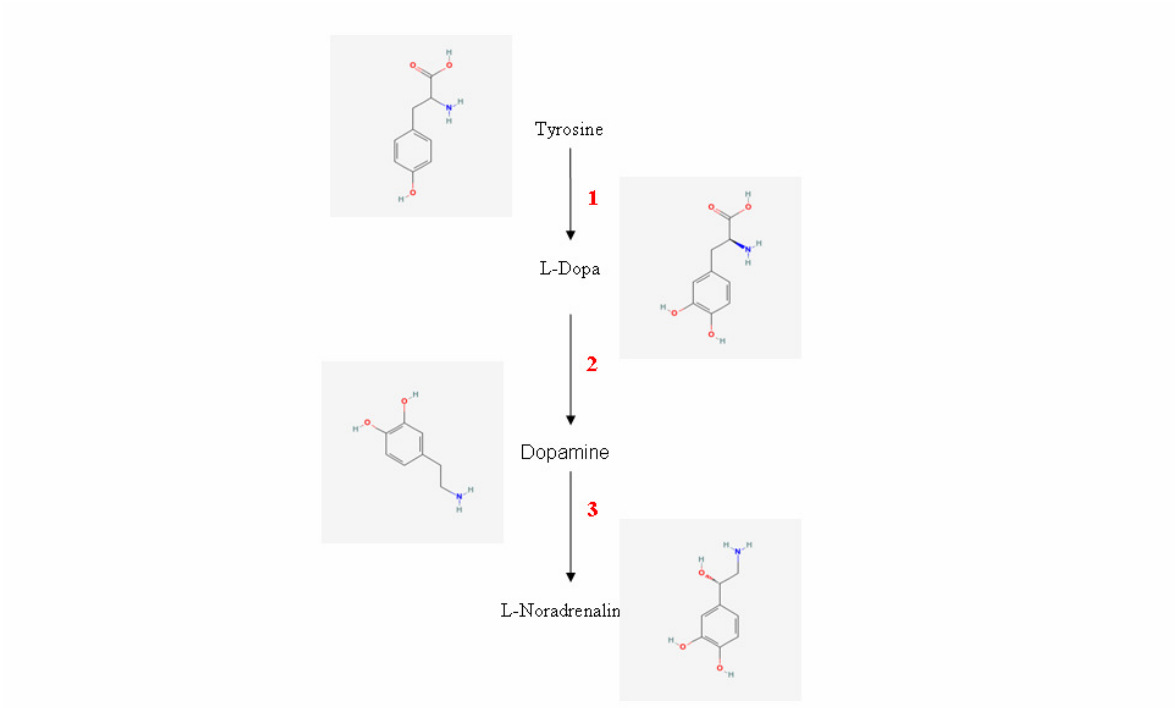


Figure 6-3: Tyrosine pathway.
Schematic representation of L-tyrosine metabolism in mammalian cells. Enzymatic steps are represented by arrows with enzymes represented by red numbers (1, tyrosine hydroxylase; 2, dopa decarboxylase; 3, dopamine beta-hydroxylase).

In dopaminergic neurons, accumulated tyrosine in converted to L-Dopa by addition of an ortho-phenolic group by tyrosine hydroxylase. Removal of the carboxyl group by dopa

decarboxylase follows. Vesicles within the neuron store and release dopamine; and when a dopaminergic nerve is stimulated the vesicles fuse with the outer membrane of the nerve (Kostrzewa *et al.*, 2005). Within the synaptic and extra-synaptic spaces dopamine is converted into inactive metabolites by the enzymes monoamine oxidase and catechol-O-methyltransferase (Mercuri & Bernardi, 2005). Clearance is also controlled by the dopamine transporter. In PD there are fewer dopaminergic nerves, meaning that fewer dopamine molecules re-enter nerve cells. Treatment involves giving exogenous L-Dopa which generates a high intraneuronal L-Dopa content, thereby elevating levels of dopamine. Combination therapy is common with L-Dopa treatment. L-Dopa is typically administered in conjunction with a decarboxylase inhibitor that is unable to cross the blood-brain barrier. This allows the exogenous L-Dopa to avoid metabolism by body tissues, leaving the most of the L-Dopa to enter the brain to produce the therapeutic effect.

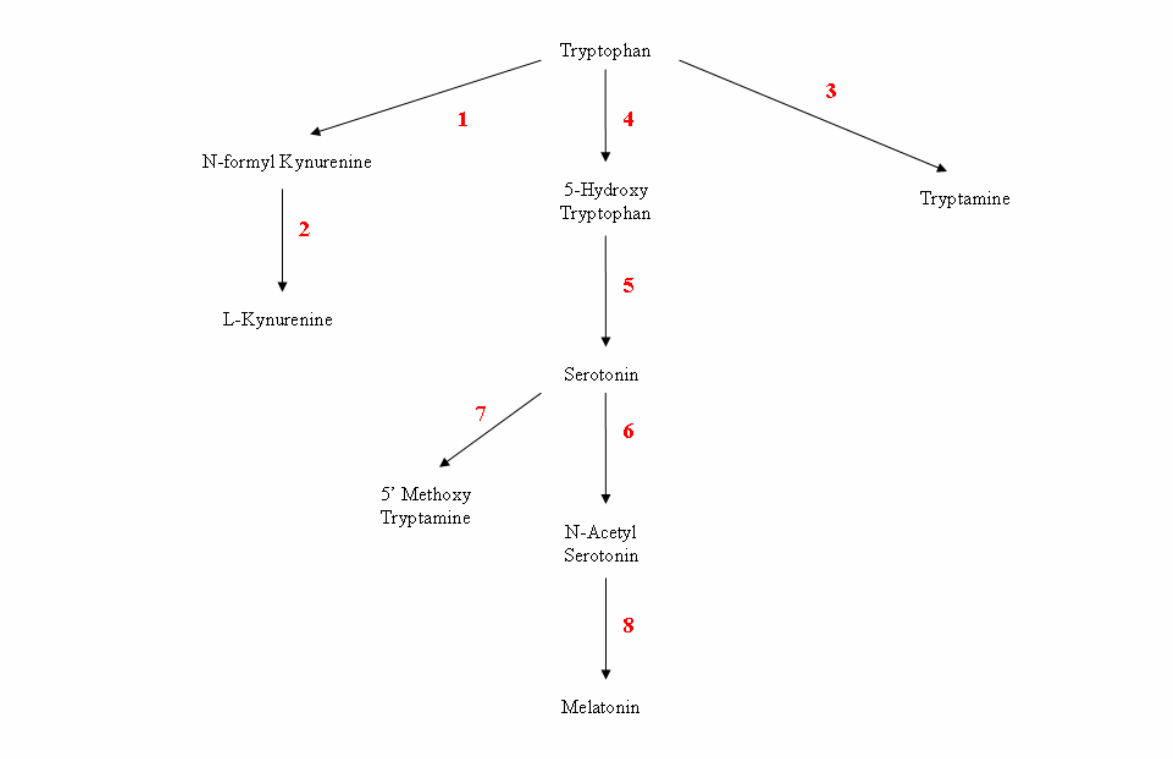


Figure 6-4: Schematic representation of L-tryptophan metabolism in mammalian cells. Enzymatic steps are represented by arrows with enzymes represented by red numbers (1, tryptophan 2,3 dioxygenase; 2, arylformidase; 3, dopa decarboxylase; 4, tryptophan hydroxylase; 5, dopa decarboxylase; 6, alkalkytransferase; 7, methyl transferase; 8, acetylserotonin O-methyltransferase).

Tryptophan is converted to 5-hydroxytryptophan via the enzymatic action of tryptophan hydroxylase (enzyme 4), with subsequent conversion to the neurotransmitter serotonin. Non-dopaminergic neurons do not normally synthesise dopamine due to the fact that they

lack the enzyme tyrosine hydroxylase. However, despite this fact, non-dopaminergic neurons can accumulate L-Dopa. In this case, the L-Dopa is converted dopamine by dopa decarboxylase.

As highlighted earlier (chapter 1.19), the range of pathophysiological effects associated with the disease are linked to trypanosome involvement, possibly by reducing host levels of essential amino acids (El Sawalhy *et al.*, 1995). Another possibility is the direct involvement of toxic or inhibitory actions of metabolites produced from aromatic amino acid metabolism in trypanosomes. The aromatic α-keto acids and indole-3-pyruvic acids have been shown to accumulate abnormally in the urine of infected mice (El Sawalhy *et al.*, 1995). This pathological symptom has been likened to other inherited disorders of aromatic amino acid metabolism. Indeed, the correlation between the physiological manifestations of trypanosomiasis and other neurological disorders cannot be discounted. It is evident that the perturbations of host environment brought about by trypanosome amino acid metabolism result in pathogenesis and physiology of the disease.

6.1.3 Cell Penetrating Peptides

Cell penetrating peptides (CPPs) have a maximum length of ~30 amino acids, have a positive charge (Deshayes, 2005), and have the potential to be novel antimicrobials These peptides are able to translocate through cell membranes without causing damage to the cell. Most research has focused on their potential use as delivery agents for therapeutic "cargo" such as DNA, peptides, and proteins; while some research focuses on their antimicrobial properties.

These peptides demonstrate an ability to translocate into cells without causing damage to the membrane (Deshayes *et al.*, 2005; Zorko & Langel, 2005). Although the specific mechanism in which the peptides cross the lipid bilayer is currently unknown, such properties have lead to the idea of using these peptides to deliver therapeutic cargo, such as DNA, peptides, or even proteins, into cells to treat various conditions and diseases (Zorko & Langel, 2005). There are no naturally occurring CPPs, most are either derived from transduction domains of native proteins, or designed to mimic the structure and sequence of such domains. Two of the best characterized CPPs are pVEC and TP10, a deletion analogue of Transportan (Elmquist *et al.*, 2001; Soomets *et al.*, 2000). Apart from uptake by mammalian cells, both peptides can successfully translocate into insect cells, protoplasts and yeast cells (Palm *et al.*, 2006; Mae *et al.*, 2005; Parenteau *et al.*, 2005). In addition to their native properties, pVEC and TP10 demonstrate antimicrobial activity at

very low concentrations (Nekhotiaeva *et al.*, 2004). This led to investigations of CPP activity on the human malaria parasite and (as outlined in this chapter) BSF trypanosomes (Arrighi *et al.*, in press).

Peptides are emerging as attractive drug delivery tools (Brooks *et al.*, 2005). The human immunodeficiency virus (HIV) encodes several trans-acting regulatory proteins not present in other retroviruses. One of these genes, designated *tat*, encodes a 14 kDa nuclear protein that acts to trans-stimulate virus gene expression and is localised predominantly in the nucleus. Recently, site-directed mutagenesis has been used to identify function domains within the tat protein (Rubens *et al.*, 1989). Tat mutations centred on the stretch of positively charged amino acids showed a retained tat function although at diminished levels. Moreover, the basic residues seemed to function as a nuclear localisation signal, a feature common among other nuclear proteins.

The tat-derived peptide is a small basic peptide that has been successfully shown to deliver a large variety of cargoes that differ in size from small particles to large nucleic acids and proteins (Brooks *et al.*, 2005). The 'transduction domain' or region conveying cell penetrating properties appears to be confined to a small stretch of basic amino acids, with the sequence RKKKRRQRRR (Ruben *et al.*, 1989). It has been shown by a structure-activity relationship study investigating which feature within the tat peptide was responsible for the membrane translocating property, that the cationic cluster of amino acids is the main functional domain determining translocation (Vives *et al.*, 1997). The common sequence determinant in most studies performed with the tat peptide is the GRKKRRQRRR sequence.

The mechanism of cell entry by the tat peptide along with other CPPs is unknown, yet several theories have been postulated from simple diffusion to an inverted micelle formation where ionic interactions between the cationic charges of the peptide and the anionic charges of the phosphate groups of the phospholipid heads of the membrane initiate the membrane absorption of the peptide (Brooks *et al.*, 2005). Membrane association occurs in an energy independent manner, although it seems energy is required for translocation across the membrane (Deshayes *et al.*, 2005).

6.1.4 Eflornithine

The ornithine analogue eflornithine (diflouromethylornithine, DFMO) was synthesised in the 1970's as a potential anticancer drug (Siimes *et al.*, 1981; Jänne *et al.*, 1981). This

inhibitor of polyamine biosynthesis acts as an irreversible inhibitor of ornithine decarboxylase (ODC), with a consequent impairment of cell division. It is thought that DFMO's mode of inhibition of ODC is by the formation of covalent adducts within the active site of the target enzyme through specific amino acid residues (Poulin *et al.*, 1992). Trypanosome cells are also sensitive to DFMO. However, the selective toxicity in treatment stems from the fact that trypanosomes are more sensitive, possibly because of the fact that polyamine depletion in trypanosomes has a greater effect than in mammalian cells. In conjunction to this, the protozoa have an ODC that is degraded and replenished much more slowly that its mammalian host (Phillips *et al.*, 1987). A 36 amino acid C-terminal region, rich in proline, glutamate, aspartate, serine and threonine residues is found in the amino acid sequence of mammalian but not *T. brucei* ODC. In eukaryotes, regions of protein with this amino acid composition is a marker for rapid intracellular degradation, meaning trypanosome ODC has a much longer half life than its mammalian counterpart (Phillips *et al.*, 1987). This is proposed as a possible cause of the selective toxicity exhibited rather than increased uptake or greater affinity for the trypanosome enzyme. Indeed, *T. brucei rhodesiense* shows a greater turnover rate of ODC and this subspecies is consequently less sensitive to DFMO action (Bacchi *et al.*, 1990; Iten *et al.*, 1995).

Uptake of this analogue of ornithine has been proposed to occur via passive diffusion although some evidence for energy dependent transport exists (Bacchi *et al.*, 1983; Bitonti *et al.*, 1987). Uptake of DFMO in *T. brucei* has previously been studied in some detail (Bitonti *et al.*, 1985). The rate of accumulation of DFMO is strongly dependent on the extracellular concentration, which suggested simple passive diffusion at play. Saturation of the uptake process with extracellular DFMO concentrations between 5 μM and 10 mM could not be demonstrated. Apparent uptake did not show inhibition by any common analogues (ornithine, lysine and arginine). However, the data presented by Bitonti shows only accumulation of DFMO at a 60 min time point, a time point which is far too long to report exclusively upon transport processes, especially when a diffusion component could also be at play. Moreover, inhibition studies only used concentrations of competition barely 20 times that of DFMO (56 μM DFMO vs 1 mM analogue). Net internalisation of compounds comprises of transport as well as downstream effects related to metabolism and distribution. Therefore, at the longer time points these latter effects play a larger role on net uptake than initial transport. Temperature sensitivity of uptake gives the most evidence for transport processes at play. Indeed, in PCF trypanosomes uptake has been shown to be temperature sensitive and, moreover, follow Michaelis-Menten kinetics with an apparent K_m of 244 μM (Phillips & Wang, 1987).

Certain biochemical changes to PCF trypanosomes associated with resistance to DFMO have been seen (Bellofatto *et al.*, 1987). DFMO resistant cell lines were created and uptake of [^{3}H]-DFMO was investigated in comparison to wild type cell lines. The rate of DFMO uptake and incorporation was found to be significantly less in resistant lines (2.5 fold less) with intracellular concentrations of DFMO calculated to be 47 µM in wild type cells and only 20 µM in resistant cell lines after 70 mins. They concluded that, in this case, the resistance phenotype was not a direct result of increased ODC activity, or a change to the inhibition profile of DFMO binding to ODC, or amplification of the ODC gene. However, increased levels of ODC mRNA were seen suggesting increased transcription resulting in the resistance phenotype seen. Although, this result seems dubious as protein extracts showed no increase in ODC levels.

DFMO also shows a profound effect on morphology of trypanosomes as well as their biochemical characteristics; causing rapid depletion of intracellular polyamines with a compensatory increased uptake of exogenous polyamines (Alhonen-Hongisto *et al.*, 1980), blocking of cytokinesis leading to multinucleated cells, alterations in macromolecular synthesis, and abnormal morphology including a broad appearance with a short free flagellum (Bacchi *et al.*, 1983). DFMO exposure of trypanosomes results in increased arginine and polyamine uptake as well as increased AdoMet decarboxylase activity (Porter *et al.*, 1987). Arginine is a precursor of ornithine and the increased exogenous polyamine uptake could be a compensatory switch for the fall in intracellular levels due to ODC inactivity.

When trying to decipher a mechanism of drug resistance that doesn't involve the target enzyme being modified or upregulated, transport defects frequently relate to resistance. In the case of DFMO, energy dependent and independent processes have both been implicated (transport versus simple passive diffusion). Assuming that both are relevant for getting DFMO into the cell only the loss of a transport system would lead to a resistance phenotype. Rapid drug efflux doesn't seem to be a mechanism in play in terms of resistance to DFMO (Bellofatto *et al.*, 1987).

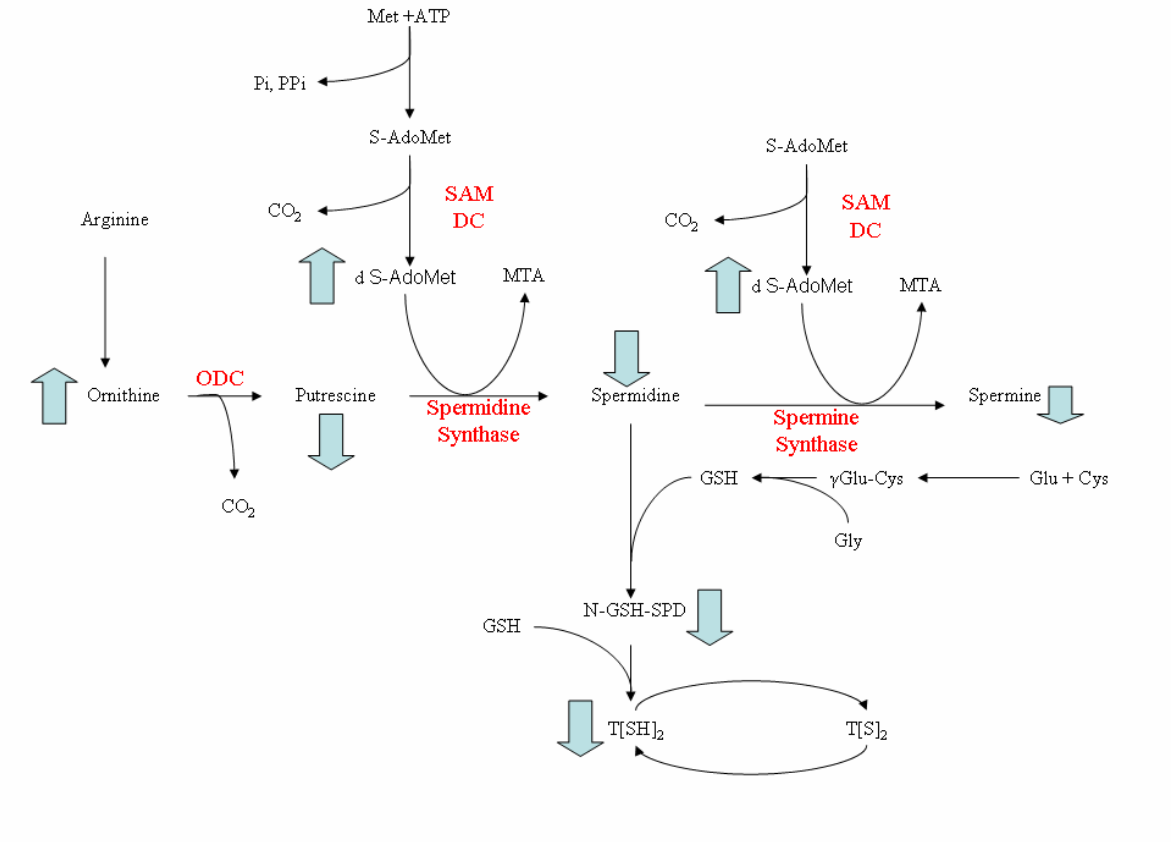


Figure 6-5: General scheme for polyamine pathway in trypanosomes.
Relevant enzymes are depicted in red. Enzymatic steps are represented by arrows. Blue arrows denote levels upon action of Eflornithine. Enzymes: ODC, ornithine decarborxylase; SAM DC, S-Adenosyl-methionine decarboxylase; spermidine synthase, spermine synthase. Abbreviations: MTA, methylthioadenosine; S-AdoMet, S-adenosyl methionine; d S-AdoMet, decarboxylated S-adenosyl methionine; Glu, L-glutamate; Cys, L-cysteine; GSH, glutathione; N-GSH-SPD, glutathionyl spermidine; $T[SH]_2$, trypanothione (reduced); $T[S]_2$, trypanothione (oxidised).

Targeted metabolite analysis revealed that exposure of parasites to the drug leads to an increase in ornithine levels and decreases of spermidine, putrescine and trypanothione, while levels of decarboxylated S-adenosylmethionine, the aminopropyl group donor in the polyamine biosynthetic pathway, also increase (Fairlamb *et al.*, 1987). One of the biochemical effects of DFMO treatment is complete elimination of putrescine and a significant reduction of spermidine (Bacchi *et al.*, 1983). Fairlamb *et al* proposed that significant polyamine depletion upon DFMO treatment effects levels of trypanothione (N^1, N^8-bis(glutathionyl)spermidine). Studies performed investigated the levels of metabolites involved in the synthesis of trypanothione using HPLC methods to detect and analyse separated thiols. The effect of DFMO treatment was to rapidly reduce putrescine levels, spermidine reduction was more gradual over the 48 hour time period, and a consequent increase of intracellular levels of ornithine (18-fold increase over 12 hours), SAM (75-fold

increase after 72 hours) and dSAM (92-fold increase after 48 hours). The consequent drop in polyamine levels is what leads to the subsequent reduction in thiol levels. Decreased levels of trypanothione result in increased sensitivity to organic peroxides such as hydrogen peroxide.

DFMO's cytostatic action requires a host response for effective elimination of parasites (Bitonti *et al.*, 1986). Indeed, parasite respiration decreases (Giffin *et al.*, 1986), and DNA synthesis is arrested (Bacchi *et al.*, 1983). Moreover, a number of morphological changes are associated with DFMO action. BSF trypanosomes after 36 hour DFMO treatment displayed characteristics similar to the stumpy form of the parasite, with multi-nucleated parasites seen (Bacchi *et al.*, 1983).

6.2 Results

6.2.1 Amino Acid Analogue Screens

The effect of a number of amino acid analogues on PCF trypanosomes as well as BSF trypanosomes was tested. Screening of 96 amino acid analogues have given several potential trypanocidal agents with varying degrees of efficacy (Table 10, Appendix 8.14). IC_{50} values of all were determined for both the PCF and BSF of the parasite. A comparative assay against the mammalian cell line (HEK 293T) was done as a preliminary for potential drug application.

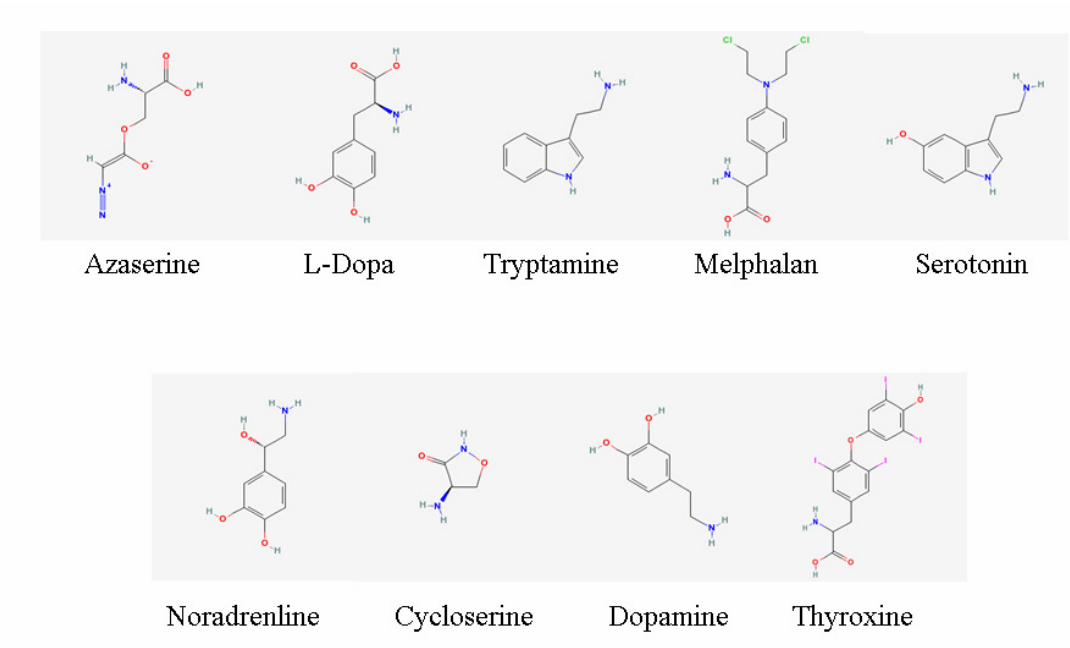


Figure 6-6: Structures of some of the relevant amino acid analogues used.

Lead candidate investigations proceeded to look at each in further detail by trying to follow the path into the cell starting at a route of uptake with respect to amino acid transporters. Furthermore, a possible cellular target involving amino acid usage was investigated. However, it is also important to note that, the action of certain amino acid analogues might be brought about by metabolic and/or extracellular activation.

Amino Acid Analogue	IC_{50} (µM)		
	PCF	BSF	HEK
Azaserine	176.46	10.85	1.52
L-Serine ß-naphthylamide	38.60	43.71	179.50
Tryptophan Benzyl Ester	96.54	27.54	N/A
Levodopa	58.58	31.58	112.21
Cycloserine	116.15	308.00	176.42
N-Benzoyl-L-Propyl-L-Tyrosine p-nitroanilide	46.20	17.40	110.00
L-Leucine ß-Naphthyl Amide	69.66	52.70	N/A
3,4 Dihydrophenylacetic acid	65.20	404.00	112.40
Tryptamine	21.90	57.83	176.42
Dopamine	63.25	275.00	87.00
Tri-Iodothyronine (T3)	134.44	102.17	N/D
L-Thyroxine	N/A	56.17	N/D
Melphalan	N/A	102.24	N/D

Table 11: Trypanocidal activities of lead candidate amino acid analogue.
Mean IC_{50} values calculated for activity against the PCF, BSF trypanosome and the HEK cell line (N/A = no activity up to 200 µM, N/D = not determined) ($n \geq 3$).

Lead candidates chosen were those that showed an IC_{50} value of less than 200 µM. The amino acid analogues show a wide range of activities; with azaserine being the most potent against the BSF of the parasite and tryptamine being the most effective against the procyclic form. The analogues azaserine and levodopa were chosen for further analysis.

The protocol for the alamar blue assay used here is adapted from Raz *et al.* (1997), using a starting BSF cell density of 10^4 cells per well. However, some labs commonly employ cell numbers as low as 10^3 cells per well. There is some evidence that the difference in starting densities does result in a shift in IC_{50}'s. This shift could be many orders of magnitude. With that in mind, it is possible that the moderate IC_{50}'s shown here by some analogues could show higher activities under different conditions.

6.2.2 Trypanocidal Activities

A killing phenotype was investigated with some of the trypanotoxic amino acid analogues. For this, PCF trypanosomes were incubated with 1 mM drug (azaserine, L-Dopa, serine ß-napthylamide, tryptophan benzyl ester and tryptamine) and viewed periodically noting their morphology.

With tryptophan benzyl ester and serine ß-napthylamide, PCF trypanosomes were killed overnight, with no dead intact parasites seen; just disintegrated cell debris. With L-Dopa killing occurred over 24 hours with dead whole cells visible leading to disintegration of cells. With L-Dopa, the medium colour changes to a heavy brown, almost black appearance. With azaserine, killing occurred much more slowly. After 48 hours some live parasites were still visible, suggesting that azaserine has a trypanostatic effect on cells rather than a trypanocidal one.

This killing phenotype was repeated for BSF trypanosomes. With tryptophan benzyl ester and serine ß-napthylamide cells were all dead within 6 hours of drug exposure. For tryptophan benzyl ester cell debris and no intact parasites were seen. Serine ß-napthylamide showed some intact dead cell (skeletons). Azaserine exposure showed a slower effect. Some cells were still alive after 6 hours and even 24 hours. Cell debris was seen as well as parasite skeleton. Killing due to levodopa was also within the first 6 hours of exposure. The killing phenotype was the most distinct of the amino acid analogues tested, with all dead cells still intact.

In BSF trypanosomes we also wanted to distinguish between trypanostatic and trypanocidal drug actions for azaserine and L-Dopa. For this, BSF trypanosomes at a starting density of 7 x 10^4 cells/ml were incubated in the presence of drug at twice its IC_{50} concentration. Cells were monitored by microscopy periodically and cell numbers were determined using an improved Neubauer haemocytometer (counting chamber; Weber Scientific).

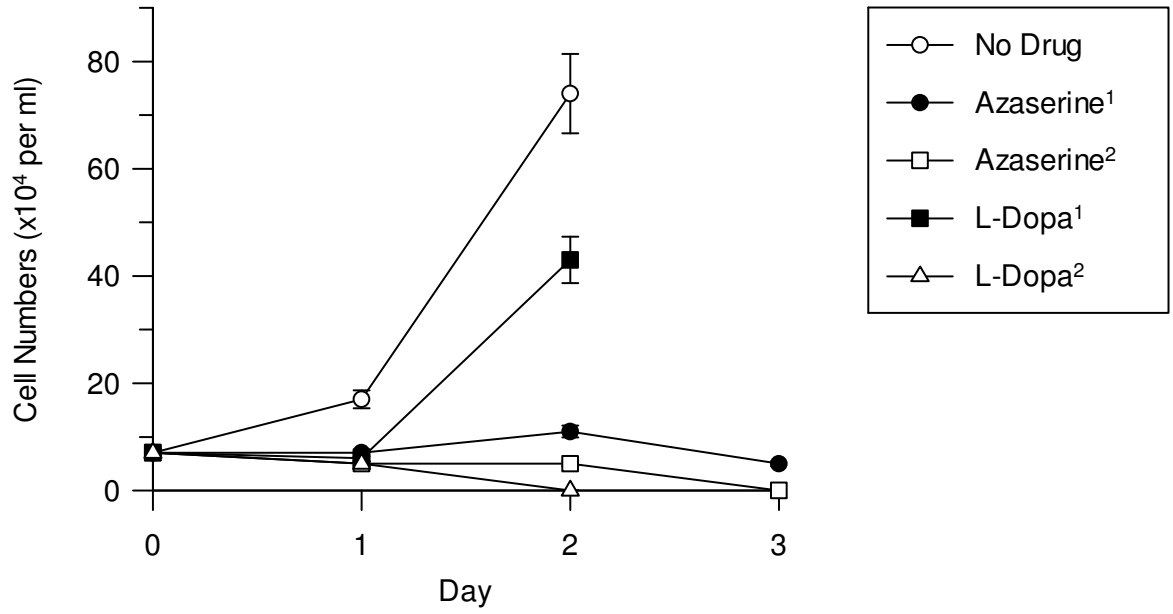


Figure 6-7: Trypanocidal cell counts.
BSF trypanosomes incubated at $1xIC_{50}$ and $2xIC_{50}$ of each drug (10 µM and 20 µM azaserine; 30 µM and 60 µM L-Dopa) over a 72 hour period (this experiment represents 3 similar repeats).

With L-Dopa at $2xIC_{50}$ concentration a trypanocidal effect was seen. Cells incubated at $1xIC_{50}$ could overcome the initial drug exposure and were seen to proliferate after 24 hours. With azaserine, a more trypanostatic effect was seen. Both concentrations of the drug used stopped trypanosomes from proliferating and only after day 3 was cell death seen.

6.2.3 Mode of action

With amino acid analogues showing high structural similarity to the naturally occurring amino acids, it is possible that a specific action involving amino acid usage is involved. Azaserine shows similarity with the amino acid glutamine and levodopa shows similarity to tyrosine. Here, we simply incubated bloodstream form trypanosomes with high concentrations of all the 20 amino acids at twice each drug's IC_{50} to determine whether any amino acids, at high concentrations, could antagonise analogue activity.

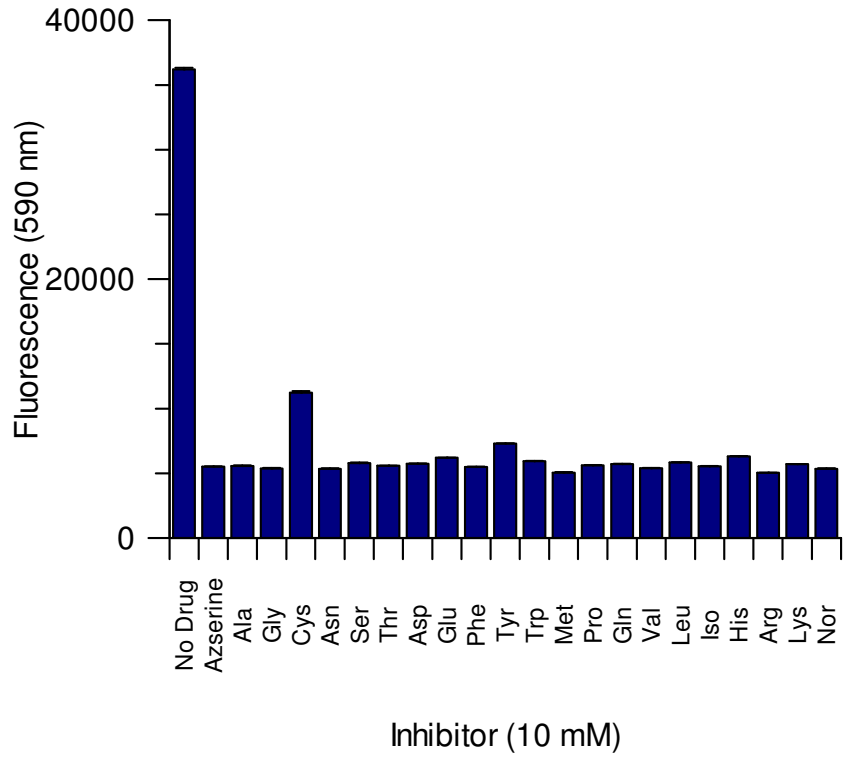


Figure 6-8: Amino acid recovery of azaserine action.
The action of 20 µM azaserine in the presence of 10 mM of each of the 20 amino acids (norvaline included), with no drug used as a control (n=3, Error bars = ±SEM).

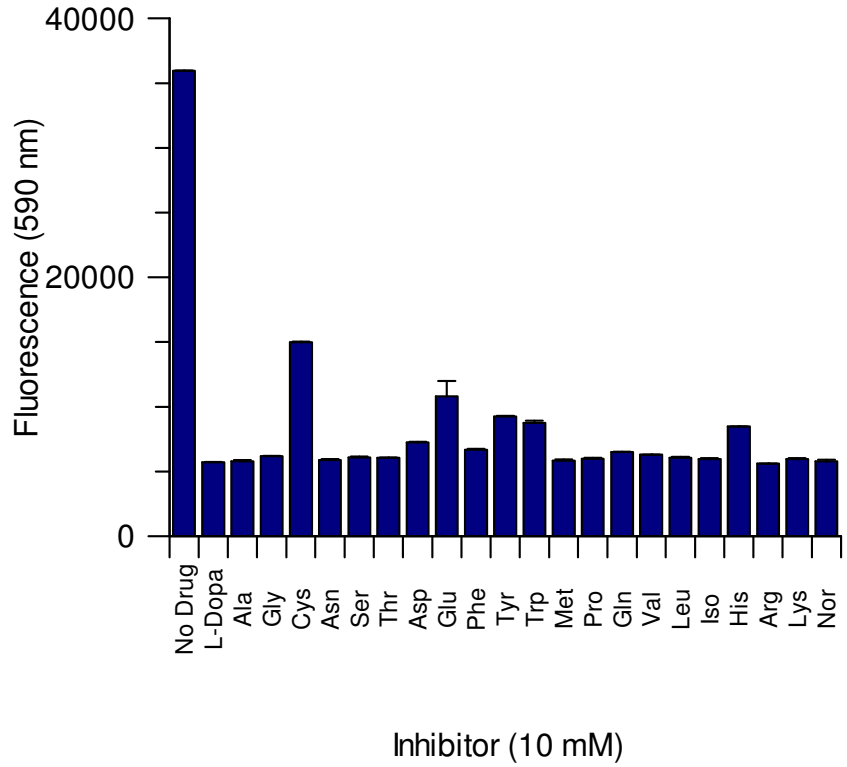


Figure 6-9: Amino acid recovery of L-Dopa action.
The action of 60 µM L-Dopa in the presence of 10 mM of each of the 20 amino acids (norvaline included), with no drug used as a control (n=3, Error bars = ±SEM).

Results showed no difference in toxicity with increased concentrations of amino acids, suggesting that the analogue's mode of action does not involve amino acid usage by trypanosomes or, at the very least; the mode of action is not competitive with other amino acids. The apparent recovery by L-cysteine was anomalous as viewing of cells by microscopy showed no live parasites.

It is possible that the high level of L-Dopa toxicity seen by Owolabi *et al* is simply as a result of metabolic activation. However, this possibility was investigated by testing all intermediates and ultimate products of the pathway for trypanocidal activity against trypanosomes.

Amino Acid Analogue	IC_{50} (µM)		
	PCF	BSF	HEK
5' Hydroxy-L-Tryptophan	N/A	N/A	N/A
Serotonin	155	N/A	N/A
Tryptamine	21.90	57.83	176.42
Dopamine	63.25	275.00	87.00
Kynurenine	N/A	N/A	N/A
Noradrenaline	N/A	N/A	N/A

Table 12: Trypanocidal activities of metabolic downstream analogues.
Mean IC_{50} values calculated for activity against the PCF, BSF trypanosome and the HEK cell line (N/A = no activity up to 100 µM, N/D = not determined) ($n \geq 3$).

Dopamine, noradrenaline, kynurenine, 5'hydroxy-tryptophan and serotonin did not show any significantly higher toxicity against trypanosomes when compared to L-Dopa and Tryptamine (Table 12). A mode of action for L-Dopa involving the generation of reactive oxygen species has already been postulated (Olowabi *et al.*, 1989). However, it was not clear if its action was due to internal or exterior activity of L-Dopa itself or one or more of its derivatives. In trypanosomes, protection against oxidative stress is controlled partly by super-oxide dismutase, a family of antioxidant metalloenzymes which detoxifies superoxide anions (Prathalingham *et al.*, 2007). We also tested L-Dopa for activity against TbSOD mutants to see if increased L-Dopa activity was evident in TbSOD knock-out lines. No difference was found in L-Dopa activity as compared to controls. L-Dopa alamar blue assays against TbSOD knock-out lines were performed by Prof. John Kelly's lab (LSHTM, London).

In order to try and select for resistance, BSF trypanosomes were passaged in medium containing incremental amounts of drug. However, the generation of resistant lines proved problematic. For azaserine, parasites showing only a 4-fold resistance were obtained, however, constant drug pressure reduced the doubling time of parasites and stable growth could not be obtained; with parasites usually dying after several passages. For levodopa, concentrations used caused progressive darkening of the medium, with turbidity developing from the production of quinolones and other reactive oxygen species. Resistant cell lines could not be generated due to the fact that trypanocidal activity may not necessarily be as a result of the analogue itself, but secondary products of the analogue.

6.2.4 Azaserine & the L-glutamine Transport System

Internalisation of amino acid analogues via amino acid transporters was an essential area we wanted to investigate. For this, we tested uptake of radiolabeled amino acids in the presence of excess concentrations of amino acid analogues. A possible route of entry was discovered by inhibition of glutamine uptake (based on structural similarity) as well as a possible route of entry through the aromatic amino acid transport system.

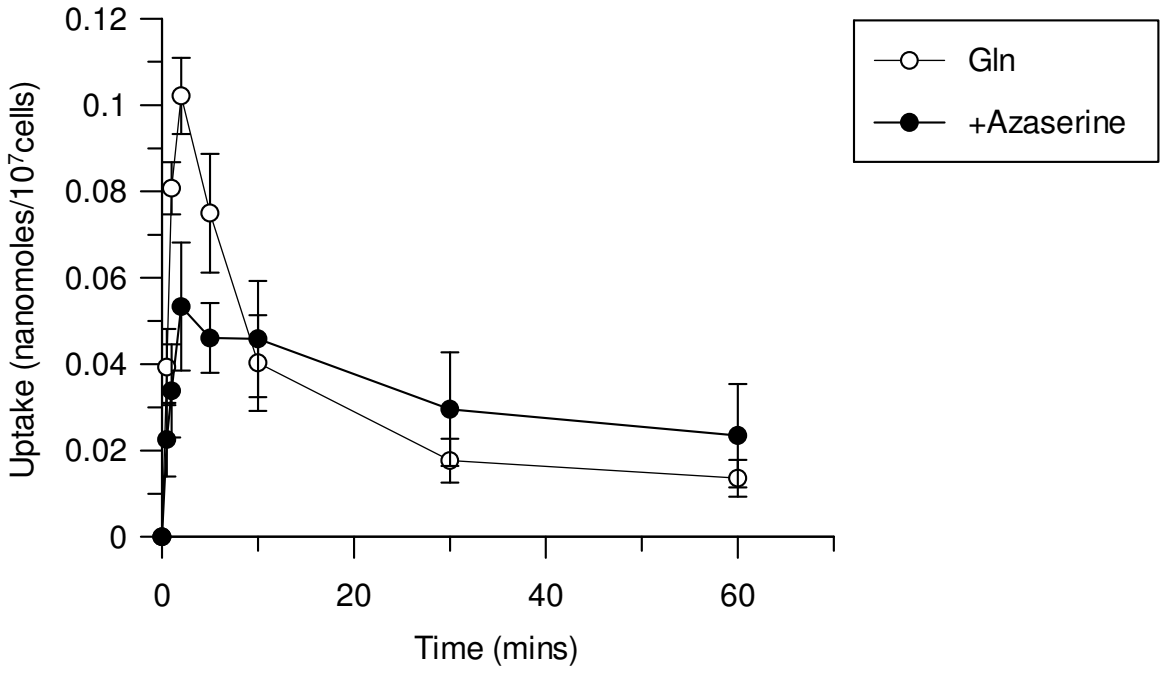


Figure 6-10: Glutamine uptake in PCF trypanosomes in the presence and absence of azaserine.
Uptake of 10 µM L-[^{3}H] Glutamine in PCF trypanosomes over a 1 hour time period, in the presence and absence of 10 mM azaserine (n=3, Error bars = ±SEM).

Glutamine uptake was studied in the presence of 10 mM azaserine over a 1 hour time period, showing a significant inhibition of uptake over a short period of time. This inhibition was never total (~50%) and beyond 5 minutes no inhibition was apparent. Since multiple routes of entry for azaserine had been postulated in other organisms (Williams *et al.*, 1980; Kaplan *et al.*, 1959; Jacquez *et al.*, 1957), we investigated a possible alternative route of entry for azaserine. Screening of a number of radiolabelled amino acids lead us to find a common route of uptake via a phenylalanine transport system.

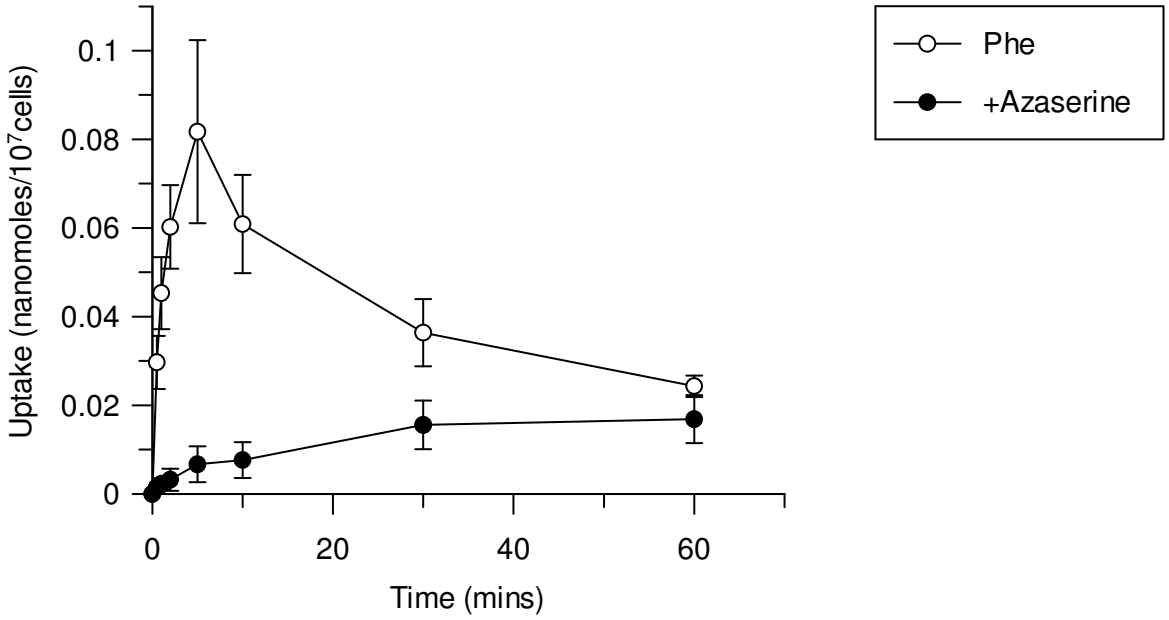


Figure 6-11: Phenylalanine uptake in PCF trypanosome in presence and absence of azaserine.
Uptake of 10 µM L-[^{3}H] Phenylalanine in PCF trypanosomes over a 1 hour time period, in the presence and absence of 10 mM azaserine (n=3, Error bars = ±SEM).

Azaserine inhibition of phenylalanine uptake is more pronounced than the inhibition seen against glutamine. Again, this inhibition is lost over time, as at the 1 hour time point, incorporation of phenylalanine in the presence of azaserine shows no comparable difference. We also wanted to investigate if azaserine inhibition of glutamine and aromatic amino acids was also evident in BSF trypanosomes.

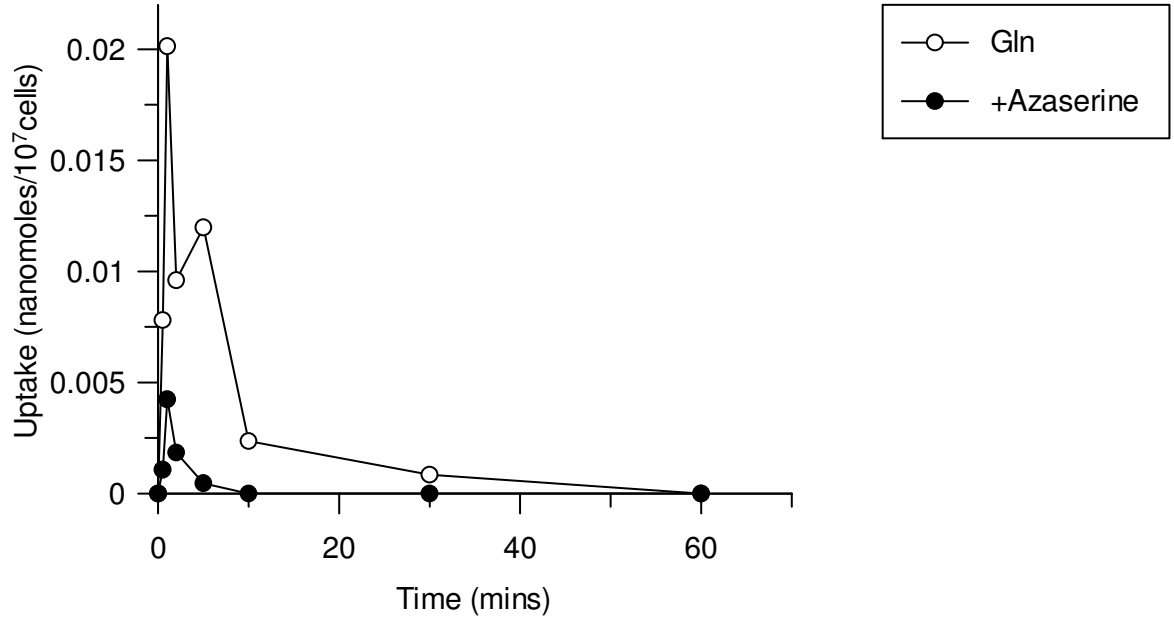


Figure 6-12: Glutamine uptake in BSF trypanosomes in presence and absence of azaserine. Uptake of 10 µM L-[^{3}H] Glutamine in BSF trypanosomes over a 1 hour time period, in the presence and absence of 10 mM azaserine (n=1).

Glutamine inhibition by high concentrations of azaserine is also seen in BSF. In this instance, glutamine inhibition seems to be to a greater degree than the inhibition exhibited by PCF trypanosomes, with 10 mM azaserine able to inhibit L-glutamine accumulation to completion.

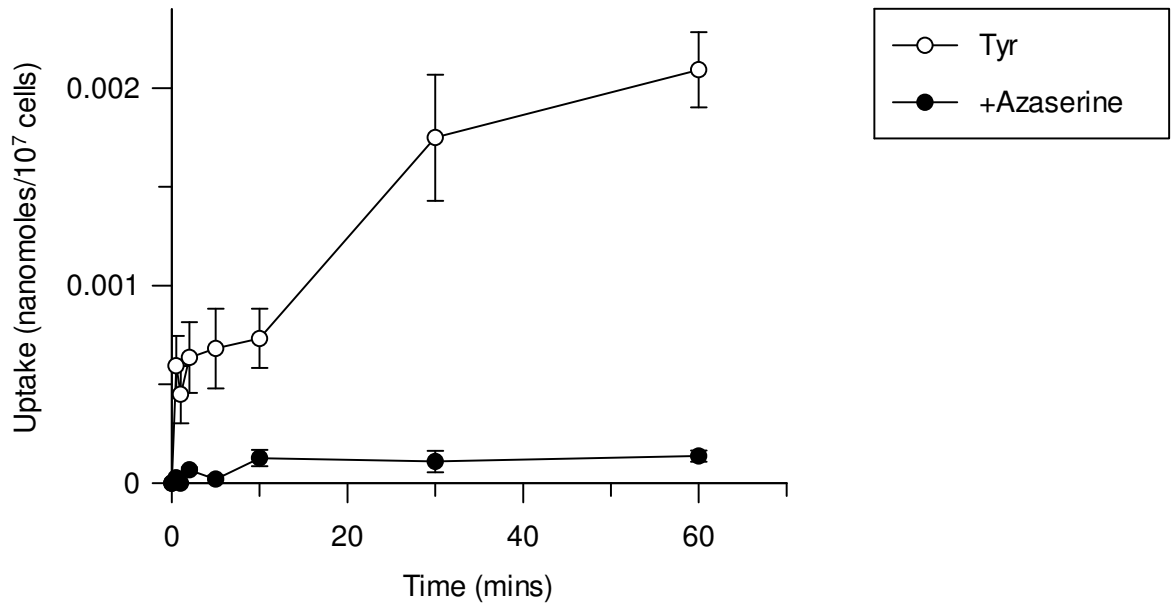


Figure 6-13: Tyrosine uptake in BSF trypanosomes in presence and absence of azaserine. Uptake of 10 µM L-[^{3}H] Tyrosine in BSF trypanosomes over a 1 hour time period, in the presence and absence of 10 mM azaserine (n=3, Error bars = ±SEM).

Tyrosine uptake in BSF trypanosomes shows an almost linear characteristic over the 1 hour time period. Internalisation of tyrosine can be inhibited by azaserine at high concentrations. Therefore, it seems evident that azaserine, at high concentrations, inhibits glutamine, phenylalanine and tyrosine internalisation by trypanosomes. We used this fact as a mode to investigate a glutamine specific transport system in PCF trypanosomes, hoping that more insight can be gained into the nature of the glutamine transport system. Firstly, to find amino acids that may be co-transported along with glutamine, inhibition of glutamine uptake was performed using all the 20 amino acids individually at a high concentration.

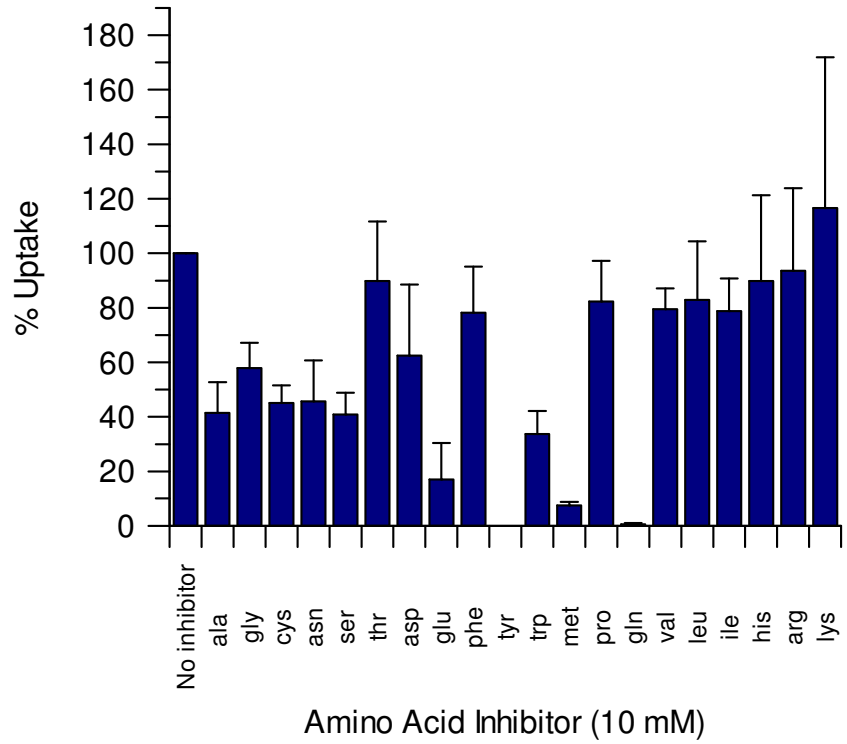


Figure 6-14: Glutamine inhibition by 20 amino acids.
Uptake of 10 µM L-[^{3}H] Glutamine in the presence of all 20 amino acids at a concentration of 10 mM. Uptake shown as a percentage of control with no inhibitor (L-Tyrosine is not soluble at a concentration of 10 mM under the conditions required for this experiment. Consequently inhibition of uptake was not determined in the presence of tyrosine) (n=3, Error bars = ±SEM).

Glutamine uptake shows high levels of competition with glutamate and methionine suggesting a shared route of entry for these three amino acids. Structural similarities proposed that this may be the case, although – functionally – glutamine shares amidic

properties with asparagine, which doesn't seem to be transported along with it. Amino acid transporters often have a broad specificity range, which sometimes can be seen as "leaky" transport. In this case, uptake of glutamine is inhibited to high levels (>90%) by a few amino acids (glutamate and methionine) whereas tryptophan shows very weak inhibition (~40%).

To determine the relative affinities of potential competitors for the transport system we used radiolabelled glutamine as the label in the presence of 0-10 mM of each of the other amino acids previously shown to inhibit glutamine uptake (Figure 6-15). Using a range of concentrations can distinguish between 'weak' and 'partial' inhibition, and can give an insight into the nature of the transport system.

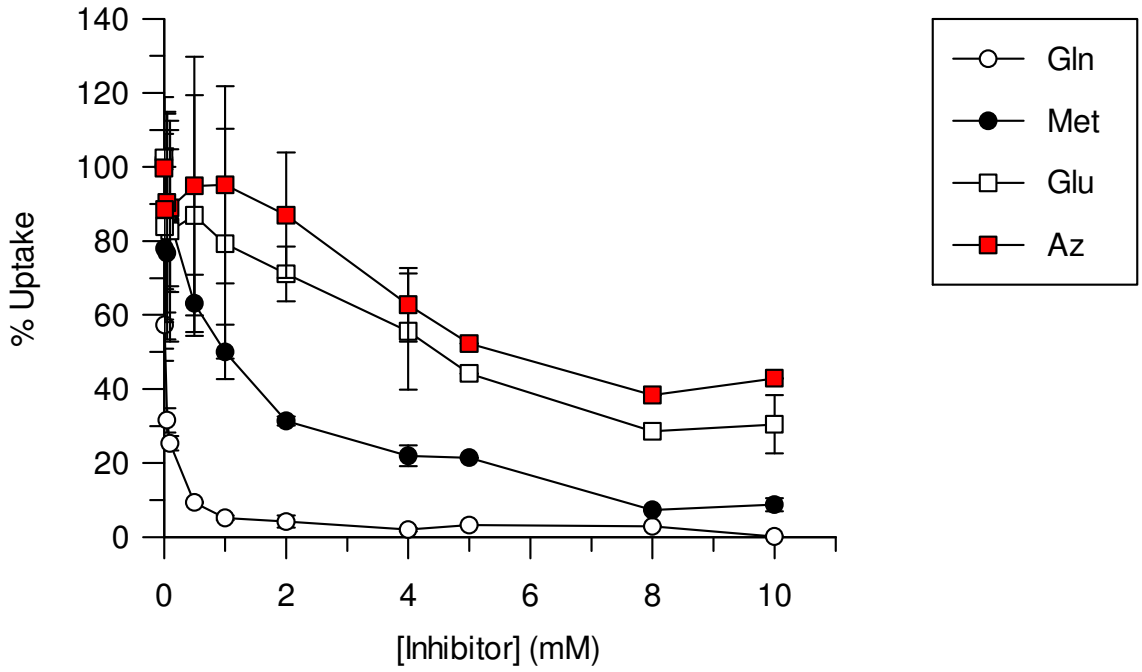


Figure 6-15: Glutamine inhibition.
Inhibition of 10 µM L-[^{3}H] Glutamine in PCF trypanosomes by cold glutamine, methionine, glutamate, and azaserine over a range of concentrations (no inhibitor, 10 µM, 50 µM, 100 µM, 500 µM, 1 mM, 2 mM, 4 mM, 5 mM, 8 mM, 10 mM) (n=3, Error bars = ±SEM).

	IC_{50} (µM)	Standard Error
Glutamine	16.03	1.1
Methionine	1393.8	326.4
Glutamate	6397.3	1938.7
Azaserine	3723.1	324.4

Table 13: Calculated IC_{50} values for inhibitors of L-glutamine uptake.

In each case these amino acids gave a progressive dose-dependent inhibition (although to different degrees), suggesting the order of affinity for a supposed transporter being glutamine>>methionine>>glutamate, with azaserine showing only partial inhibition.

The calculated IC_{50} values refer to the amount of inhibitor required to inhibit uptake of a particular amino acid by 50%. Only glutamine shows an IC_{50} value in the micro molar range, with the others in the millimolar range, suggesting that this transporter has a higher affinity for glutamine as compared to all the others.

6.2.5 Levodopa & the Aromatic Transport System

Studies here on aromatic amino acid uptake in trypanosomes have been done in the context of the amino acids phenylalanine and tyrosine, along with the tyrosine analogue, Levodopa. None of the other aromatic amino acid analogues (noradrenalin, serotonin, tryptamine, or dopamine) showed any inhibition of phenylalanine uptake (Figure 6-16).

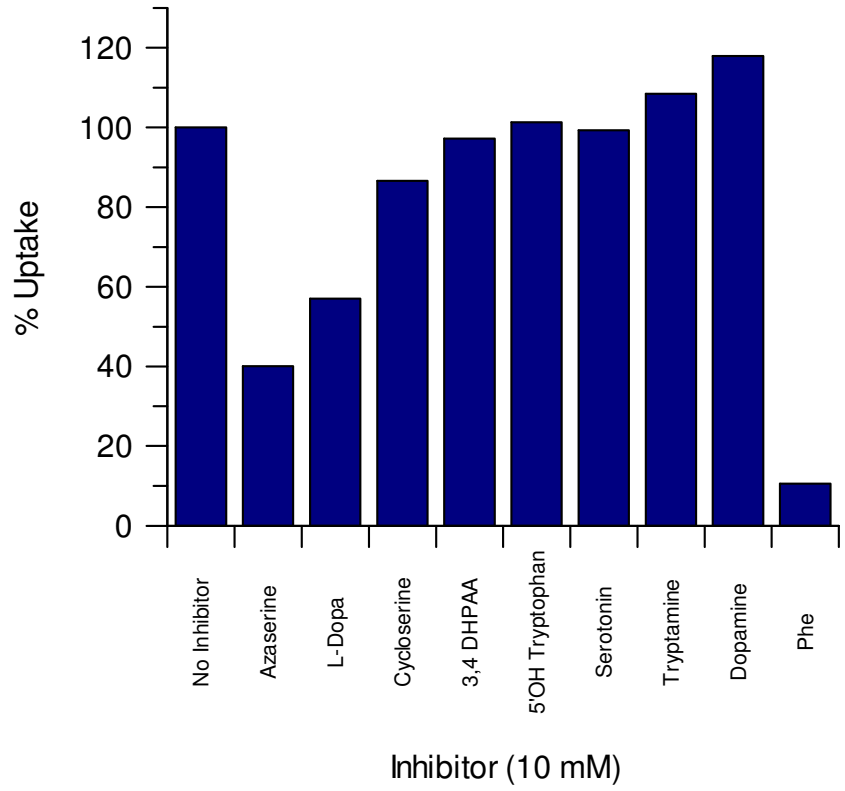


Figure 6-16: Phenylalanine inhibition by amino acid analogues.
Uptake of 10 µM L-[^{3}H] Phenylalanine in PCF trypanosomes in the presence of a range of analogues at a concentration of 10 mM (L-Dopa concentration at 1 mM) (n=1).

Levodopa's high structural similarities to tyrosine lead us to investigate its internalisation through a tyrosine specific transport system. We investigated uptake of tyrosine in PCF trypanosomes over the course of 1 hour in the presence of 1 mM L-Dopa.

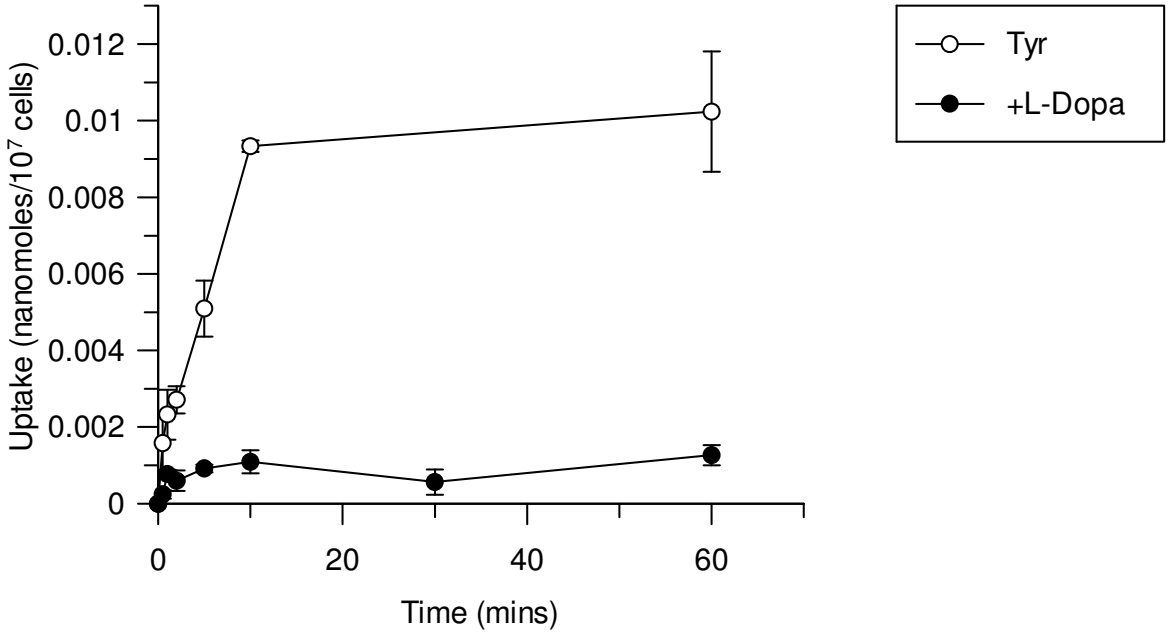


Figure 6-17: Tyrosine uptake in PCF trypanosomes in the presence and absence of L-Dopa. Uptake of 10 µM L-[^{3}H] Tyrosine in PCF trypanosomes over a 1 hour time period, in the presence and absence of 1 mM L-Dopa (n=3, Error bars = ±SEM).

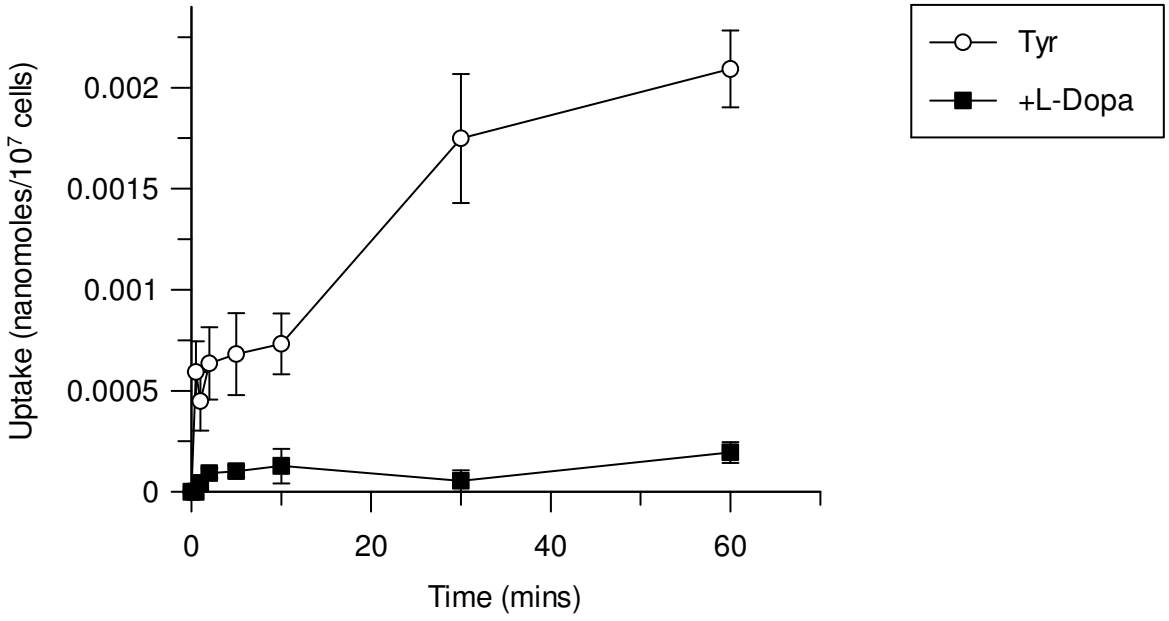


Figure 6-18: Tyrosine uptake in BSF trypanosomes in the presence and absence of L-Dopa. Uptake of 10 µM L-[^{3}H] Tyrosine in BSF trypanosomes over a 1 hour time period, in the presence and absence of 1 mM L-Dopa (n=3, Error bars = ±SEM).

L-tyrosine uptake was inhibited to a large degree by 1 mM L-Dopa, with uptake almost non-existent over the 1 hour time period. This was evident in both the PCF and BSF stage of the parasite. Given the fact that aromatic amino acids have no significant metabolic function within the cells, accumulation of amino acids within the cell seems odd. As in the previously described work with L-glutamine, inhibitor studies might give insight into structural specificities of a tyrosine transporter in trypanosomes.

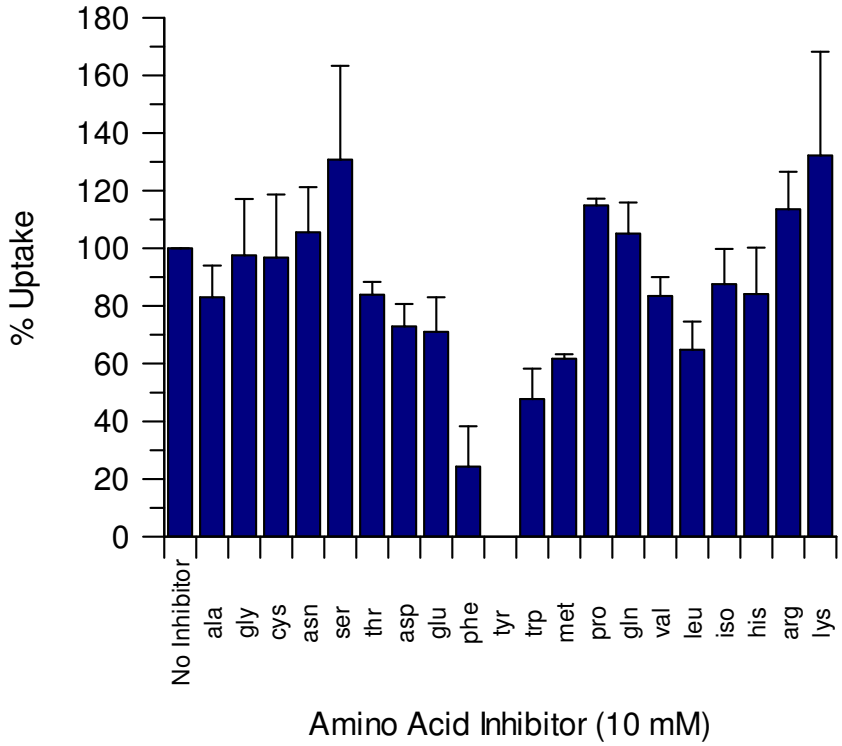


Figure 6-19: Tyrosine inhibition by 20 amino acids.
Uptake of 10 µM L-[^{3}H] Tyrosine in PCF trypanosomes in the presence of all 20 amino acids at a concentration of 10 mM. Uptake is shown as a percentage of control with no inhibitor (L-Tyrosine is not soluble at a concentration of 10 mM under the conditions required for this experiment. Consequently inhibition of uptake was not determined in the presence of tyrosine) (n=3, Error bars = ±SEM).

Tyrosine uptake is inhibited relatively poorly by the 20 amino acids at 10 mM, with phenylalanine and tryptophan the two amino acids that inhibit the most (~20-50% uptake), and methionine showing ~60% inhibition. It is possible that a common route of entry exists for the aromatic amino acids due to structural similarity, however since inhibition is never total it is possible multiple transporters play a role in uptake. Indeed, previous work investigating aromatic amino acid uptake in BSF trypanosomes suggested the existence of

several aromatic amino acid transporters (M.P. Hasne, thesis). Phenylalanine and tryptophan were found to use at least 2 routes of entry while a single high affinity transporter was identified for tyrosine.

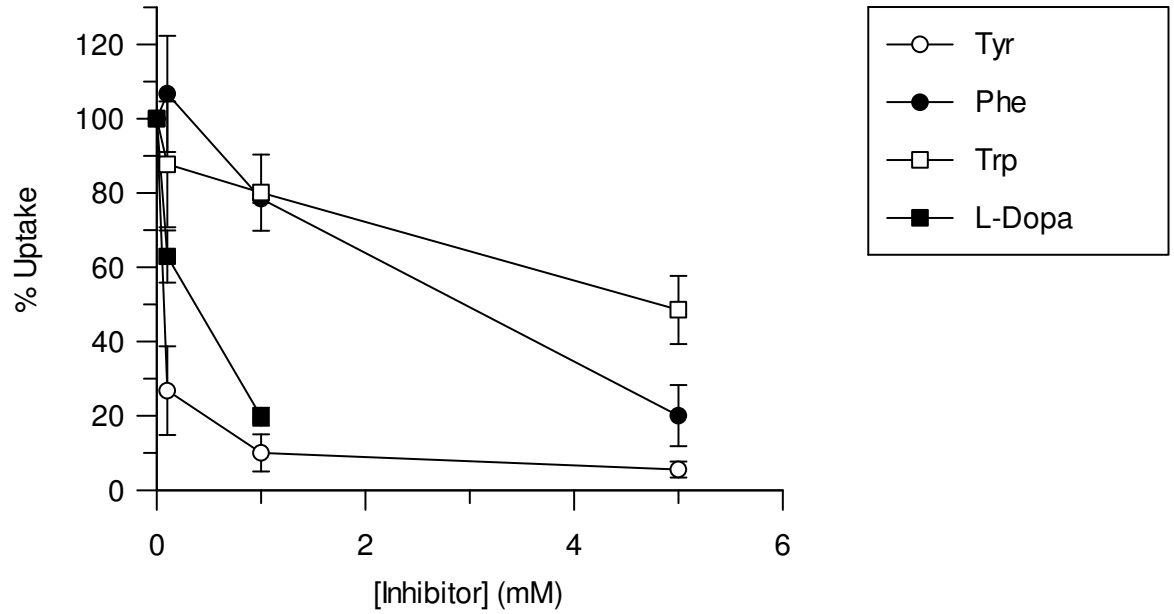


Figure 6-20: Tyrosine inhibition.
Inhibition of 10 µM L-[^{3}H] Tyrosine in PCF trypanosomes by cold tyrosine, phenylalanine, tryptophan and L-Dopa over a range of concentrations (no inhibitor, 0.1 mM, 1 mM, and 5mM). Uptake shown as a percentage of control with no inhibitor (n=3, Error bars = ±SEM).

Inhibition of tyrosine under increasing amounts of cold tyrosine, phenylalanine, and tryptophan showed a dose dependent relationship, with tryptophan being the weakest of all the aromatic inhibitors. L-Dopa was only used at a highest concentration of 1 mM due to the limits of its solubility in water. Higher concentrations of L-Dopa can be achieved with dilution in HCl, however, the hydrogen ions alter the pH of the uptake buffer and subsequently alter uptake by trypanosomes. From this, it seems possible that a tyrosine-specific transport system also shows some specificity for phenylalanine and tryptophan, and great specificity for L-Dopa.

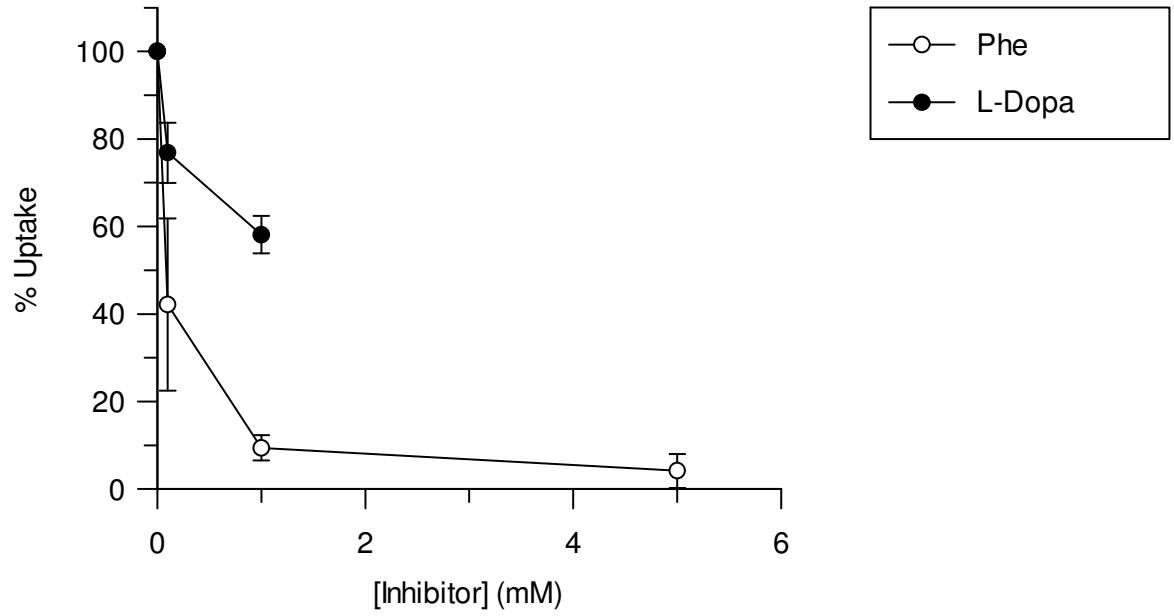


Figure 6-21: Phenylalanine inhibition.
Inhibition of 10 µM L-[^{3}H] Phenylalanine in PCF trypanosomes by cold phenylalanine and L-Dopa over a range of concentrations (no inhibitor, 0.1 mM, 1 mM, and 5mM). Uptake shown as a percentage of control with no inhibitor (n=3, Error bars = ±SEM).

Phenylalanine self-inhibition reaches completion at 5 mM. Inhibition of phenylalanine with L-Dopa at the 1 mM concentration used inhibits phenylalanine uptake by ~40%. In comparing the result here with that from the tyrosine inhibition experiment it seems that L-Dopa does have specificity for both the tyrosine and phenylalanine transport systems, although to differing degrees. If one transport system for the aromatic amino acids was evident then L-Dopa inhibition of both tyrosine and phenylalanine would be to a similar degree. The fact that more inhibition of tyrosine uptake is seen suggests that different transport systems occur for the aromatic amino acids.

6.2.6 Cell Penetrating Peptides

Peptide	Sequence	Length
pVEC	LLIILRRRIRKQAHAHSK	18
TP10	AGYLLGKINLKALAALAKKIL	21
Tat	GRKKRRQRRR	10

Table 14: CPP amino acid sequence.

Peptides compromise short amino acid chains, and there has been some interest in the antimicrobial activity of some peptides. Here we test three CPPs (TP10, pVEC and tat-derived peptide) for anti-trypanosomal activity and look at the internalisation of TP10 and tat-derived peptide.

The TP10 and pVEC CPPs were from Dr Romanico Arrighi (University of Stockholm) whereas the tat-derived peptide was from Dr Catherine Berry (University of Glasgow). We investigated CPP activity, and monitored TP10, pVEC and tat peptide activity against blood stage trypanosomes *in vitro*. Here TP10 shows strong activity, with an IC_{50} value of 2.7 µM. In contrast, pVEC was ineffective at reducing the parasite numbers up to concentrations of 25 µM. The tat-derived peptide failed to show any trypanocidal activity at concentrations up to 100 µM. In order to determine whether TP10 was lysing BSF trypanosomes, we monitored the parasites every hour up to six hours following the addition of TP10 (Figure 6-22).

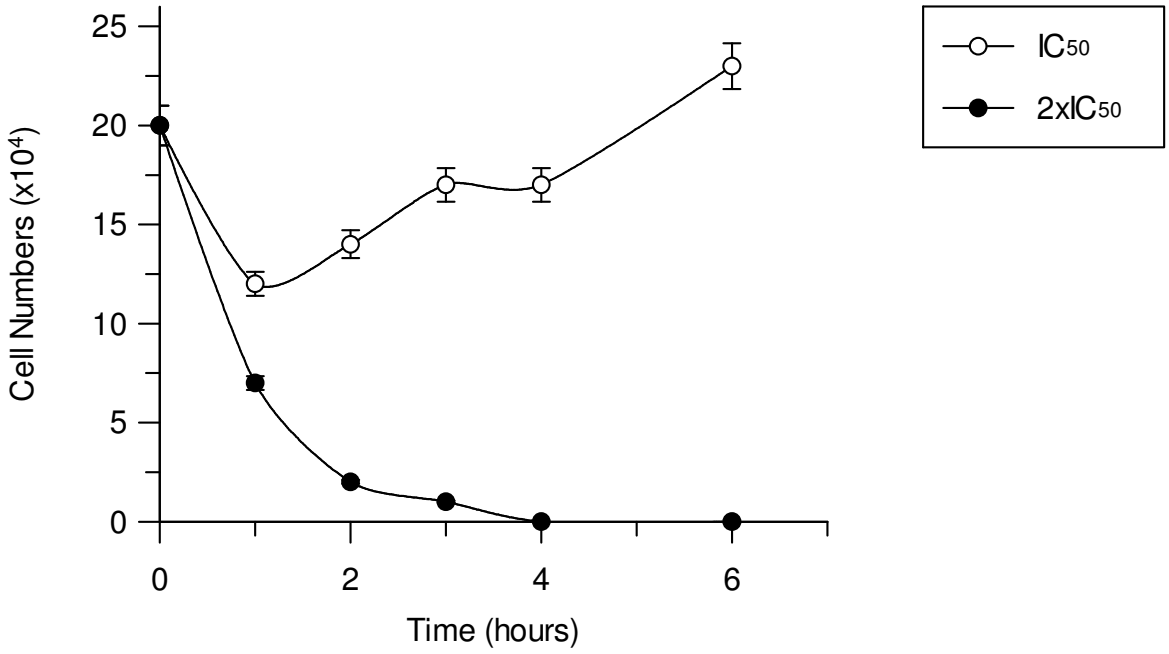


Figure 6-22: CPP activity against BSF trypanosomes.
Cell numbers viewed microscopically over a 6 hour time period (this experiment represent data from 3 similar repeats).

The parasite numbers dropped by ~20% within one hour of treatment at the IC_{50} concentration (2.7 µM). No further reductions were noted in the subsequent hours post-treatment. However at $2xIC_{50}$ (5.4 µM), an initial drop in parasite numbers was followed

by a steady decline in the parasite population, with complete cell clearance after 6 hours. This data shows that TP10 is effective against blood stage trypanosomes.

To shed light on the mechanism of TP10 trypanocidal activity we used FITC-conjugated TP10 (fluorescin isothiocyanate), and monitored potential uptake and localisation in the BSF trypanosome as compared with DAPI (4',6-diamidino-2-phenylindole, a fluorescent stain that binds strongly to DNA). BSF trypanosomes were exposed to either 10 µM or 50 µM drug. Rapid cell death was seen at higher concentrations, and even at these concentrations cell death was evident.

The compound was seen to enter dead cells. However, live cells did not show significant internalisation of the compound. And few showed co-localisation with the kinetoplast and the nucleus and others with just the kinetoplast. It is possible that greater concentrations of drug are needed to show any significant staining of the trypanosome cell interior. However, because of trypanocidal activity of the compound, it will be hard to achieve without cell death.

The internalisation of the tat peptide into the interior of the trypanosome was also investigated. Internalisation of the peptide was not seen at concentrations of up to 60 µg/ml (~43 µM), with up to 3 hours incubation time. No staining of cell interior was seen, as compared to punctuate localisation with DAPI as a control.

6.2.7 DFMO Mode of Uptake

Here, we investigate further the mode of uptake of DFMO and its relation to other amino acids with respect to uptake via an amino acid transport system. Due to short supply of ^{3}H-DFMO (a gift from Dr S Thomas, London), only a preliminary study of DFMO uptake was possible.

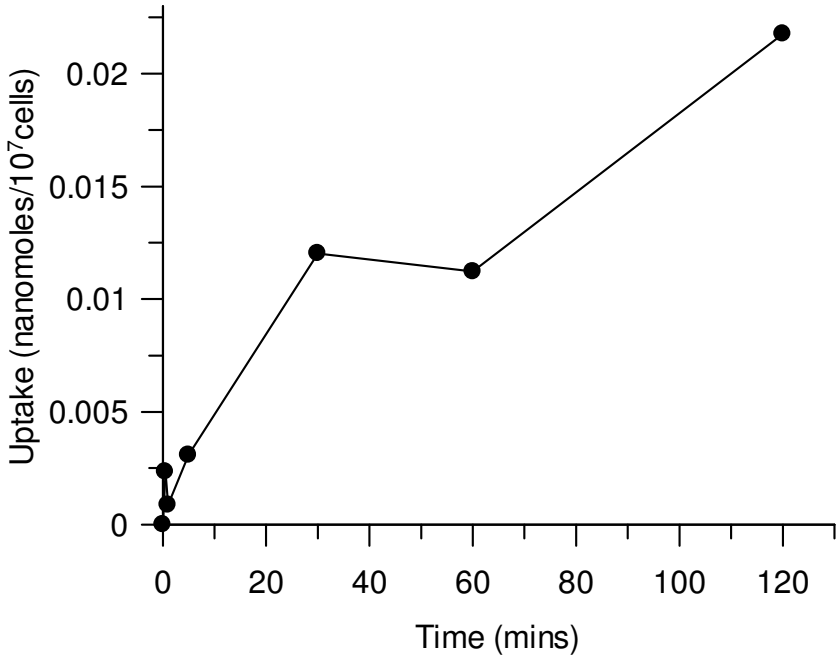


Figure 6-23: DFMO uptake.
Uptake of 10 µM ^{3}H-DFMO in BSF trypanosomes over a 2 hour time period (n=1).

Uptake of 10 µM ^{3}H-DFMO over the 2 hour time period appears to be essentially linear, showing an approximate accumulation of drug at a rate of 10 picomoles per hour (per 10^{7} cells).

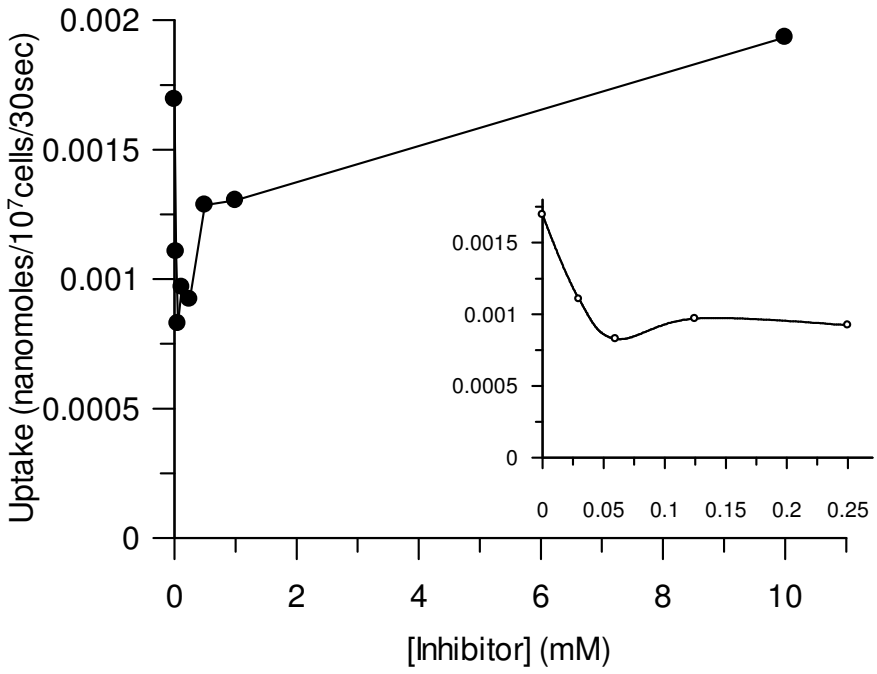


Figure 6-24: DFMO self-inhibition.
Inhibition of 10 µM ^{3}H-DFMO in BSF trypanosomes by cold DFMO over a range of concentrations (no inhibitor, 30 µM, 60 µM, 125 µM, 250 µM, 500 µM, 1 mM, 10 mM). Inset: 0 – 250 µM (n=1).

Next we wanted to determine if uptake of DFMO could be inhibited by increasing doses of unlabelled drug. Self-inhibition of DFMO never reaches a baseline level; with an apparent stimulation of uptake from 250 to 500 µM onwards. Increased DFMO concentrations seem to re-stimulate increased uptake of DFMO. An IC_{50} value was calculated (26.7 µM ± 4.6) based on the concentration which gave half of the maximal inhibition. If inhibition is incomplete, this could suggest more than one route of entry for a particular substrate. In this case, inhibition at its maximum is only 50% complete.

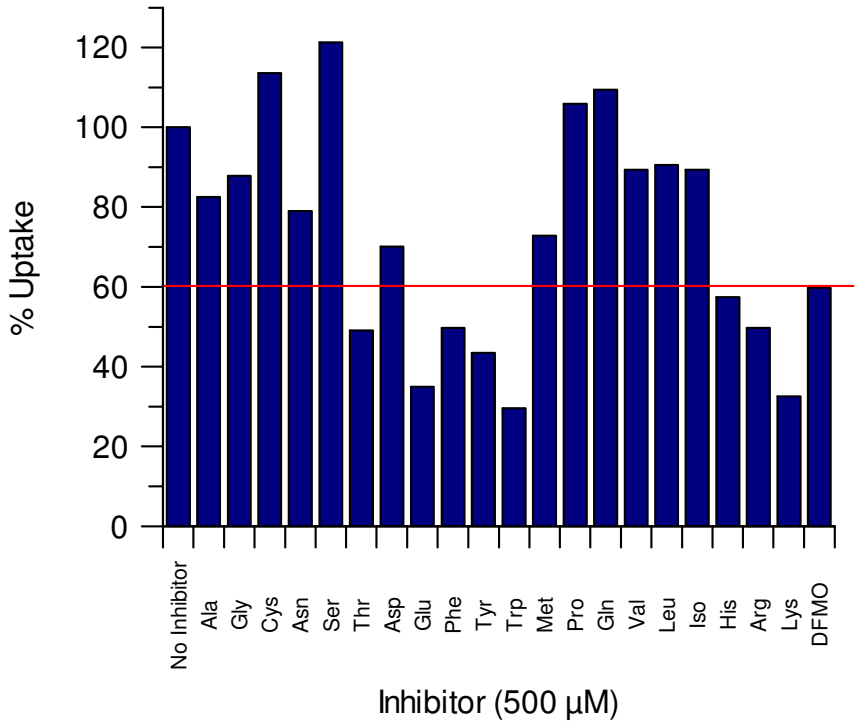


Figure 6-25: DFMO inhibition by 20 amino acids.
Uptake of 1 µM ^{3}H-DFMO in BSF trypanosomes in the presence of all 20 amino acids at a concentration of 500 µM. Uptake shown as a percentage of control with no inhibitor, with red line indicating inhibition level by cold DFMO (n=1).

Inhibition of [^{3}H]-DFMO (1 µM) by the 20 amino acids using a 500-fold excess of cold inhibitor shows that lysine, arginine, threonine, glutamate and the aromatic amino acids can inhibit DFMO uptake beyond 50%. Only a 500-fold excess was used as to avoid stimulation of another possible intake mechanism at high concentrations as noted by the data collected on DFMO self inhibition with cold DFMO. This information provides more preliminary evidence for multiple routes of entry via amino acid specific transport systems. Inhibition greater than the level of self-inhibition exhibited by DFMO on its own is shown by the amino acids threonine and glutamate, as well as two amino acid groups (the basic

and aromatic amino acids). These latter two groups were then used to study additive inhibition.

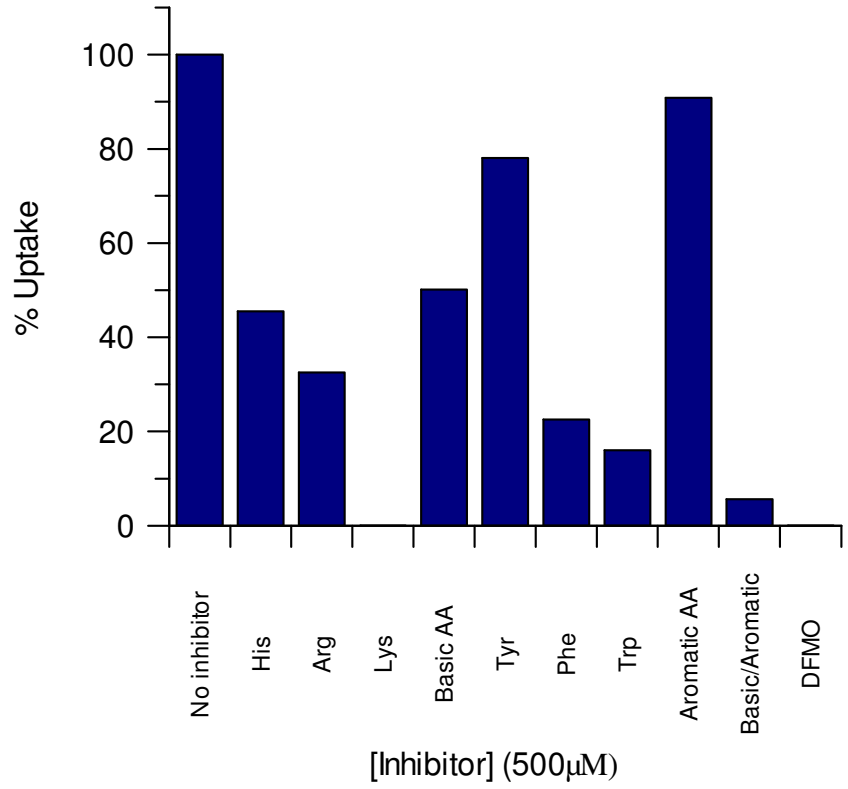


Figure 6-26: DFMO inhibition by basic and aromatic amino acids.
Uptake of 1 µM ^{3}H-DFMO in BSF trypanosomes in the presence of 500 µM amino acid inhibitors (histidine, arginine, lysine, tyrosine, phenylalanine, tryptophan, and combinations of both the basic (arginine, lysine and histidine) and aromatic (phenylalanine, tryptophan and tyrosine) amino acids at a concentration of 500 µM each) (n=1).

The basic and aromatic groups of amino acids were used to study additive inhibition using the same inhibition concentrations as previously stated (500 µM inhibitor versus 1 µM DFMO). All amino acids used showed some level of inhibition, with lysine showing total inhibition of DFMO uptake. Interestingly, high concentrations of multiple amino acids did not completely reduce uptake of DFMO. DFMO uptake in the presence of all three basic amino acids showed levels comparable with histidine on its own. Similarly, high levels of the aromatic amino acids restore DFMO uptake. If a sole basic amino acid transporter was present we would expect to see total inhibition of DFMO uptake in the presence of all the basic amino acids. However, the fact that uptake is still seen in the presence of the basic amino acids points to a few possibilities. Firstly, that multiple transporters are present, the kinetics and capacity of which cannot be distinguished from these preliminary

experiments. And secondly, it is possible that some sort of trans-acceleration effect is occurring in the presence of high concentrations of the amino acids. Trans-acceleration refers to the increased capacity of a single transporter. Thirdly, the presence of high concentrations of certain amino acids might stimulate the opening of another related transporter. This might go some way to explaining the restored uptake of DFMO in the presence of the aromatic amino acids, and is consistent with the apparent re-stimulation of DFMO uptake (Figure 6-24).

It seems possible that multiple routes of entry for DFMO are present. Lysine differs from DFMO by one CH_2 unit on its side chain and an altered amino acid recognition motif, with the hydrogen replaced by a difluoromethyl unit (Figure 6-32). It is expected that DFMO's high structural similarity to lysine will mean it shares a route of entry through a lysine specific transport system. We then went on to assess DFMO's ability to inhibit tyrosine and lysine uptake, using radiolabeled amino acids and cold DFMO at high concentrations.

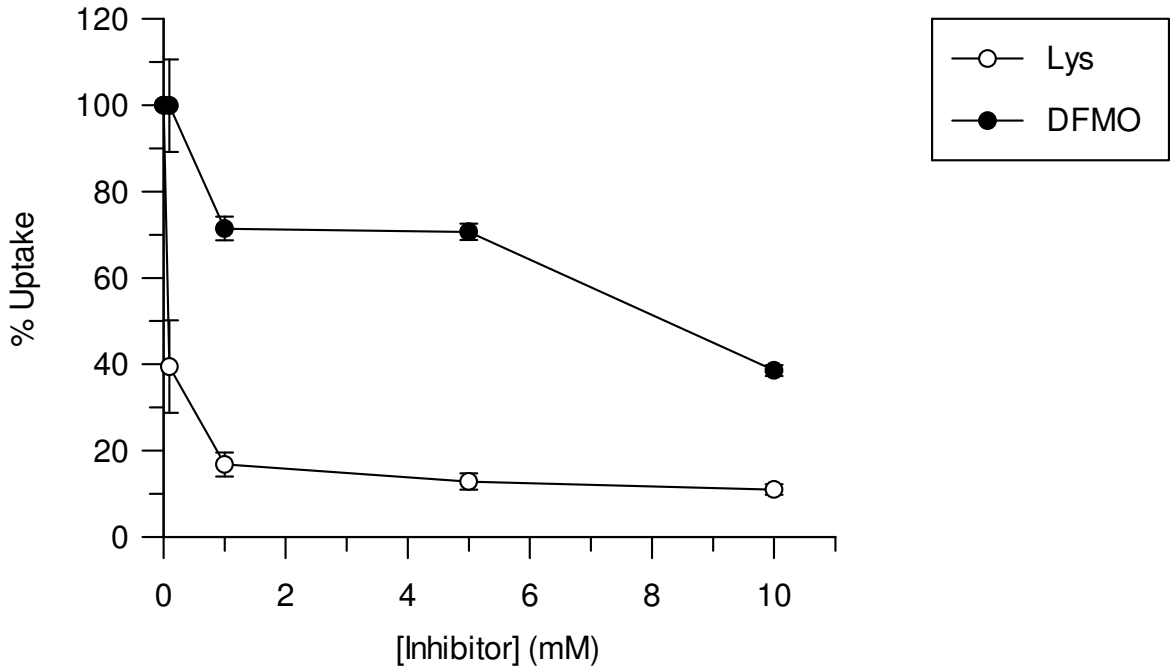


Figure 6-27: Lysine inhibition by DFMO and lysine.
Uptake of 10 μM L-[^{3}H] Lysine in BSF trypanosomes at a single 30 sec time point in the presence of cold lysine and DFMO over a range of concentrations (no inhibitor, 0.1 mM, 1 mM, 5mM). Uptake shown as a percentage of control with no inhibitor (n=3, Error bars = ±SEM).

Lysine inhibition seems dose dependent with respect to cold lysine. From the curve an apparent IC_{50} of 39 μM ± 4 was calculated. In the presence of DFMO, inhibition is seen

but is never total (~60% inhibition of 10 μM lysine at a 10 mM DFMO concentration). Lysine uptake, unlike DFMO (Figure 6-24), is not re-stimulated at higher concentrations of DFMO. If DFMO is targeted through a lysine specific transporter, the nature of this transporter, in its capacity and kinetic behaviour, is still to be elucidated. DFMO uptake via another route was explored by inhibition of tyrosine uptake.

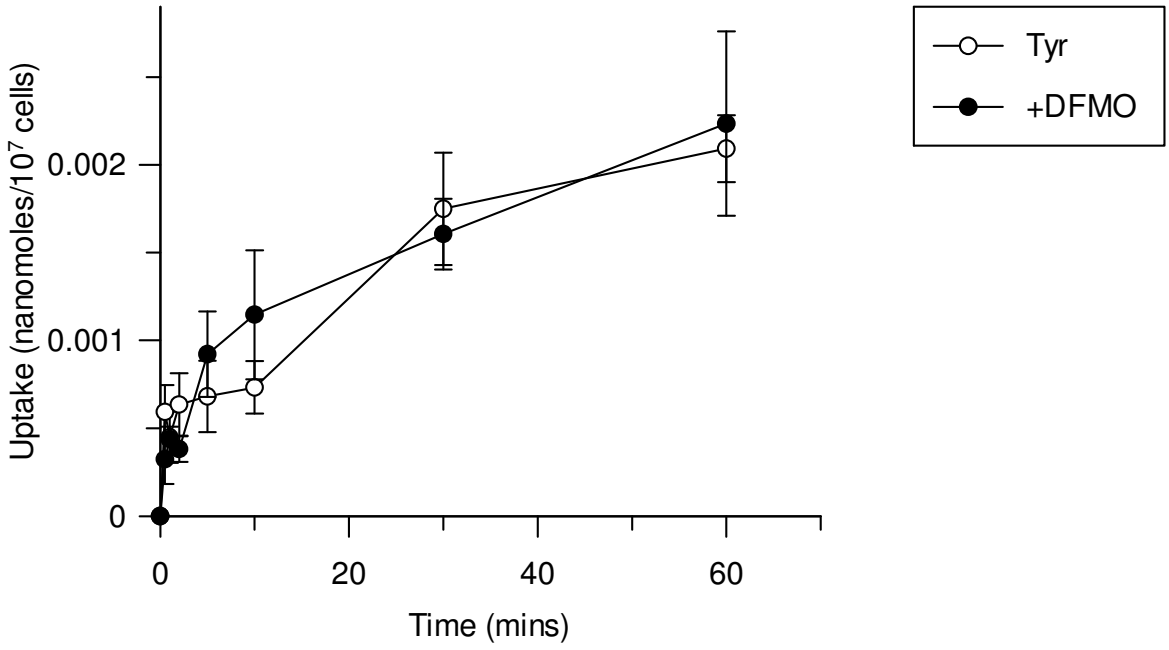


Figure 6-28: Tyrosine uptake in BSF trypanosomes in presence and absence of DFMO.
Uptake of 10 μM L-[^{3}H] Tyrosine in BSF trypanosomes in the presence and absence of 10 mM DFMO over a 1 hour time period (n=3, Error bars = ±SEM).

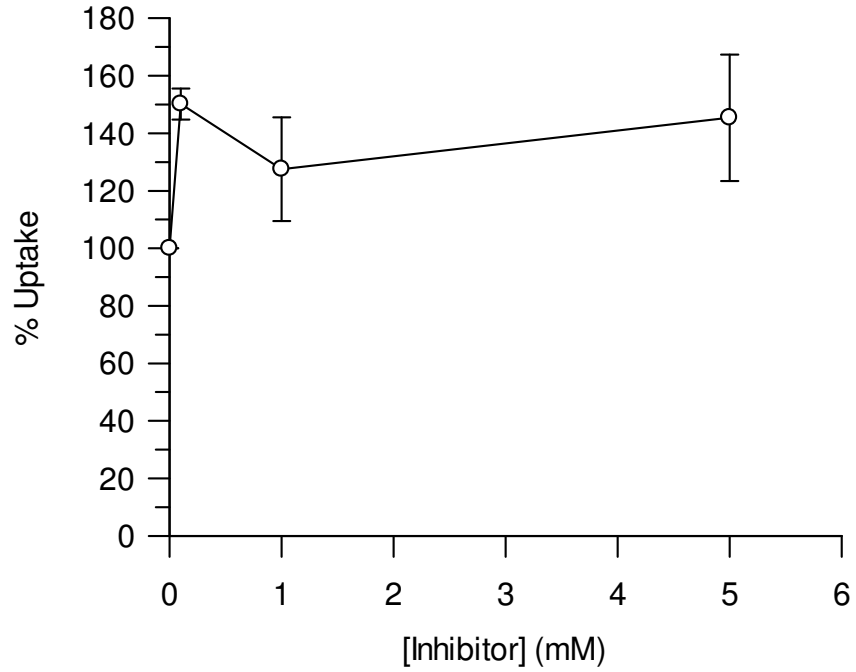


Figure 6-29: Tyrosine inhibition by DFMO.
Uptake of 10 µM L-[^{3}H] Tyrosine in BSF trypanosomes in the presence of DFMO at a range of concentrations (no inhibitor, 0.1 mM, 1 mM, and 5 mM). Uptake shown as a percentage of control with no inhibitor (n=3, Error bars = ±SEM).

Tyrosine uptake in the presence of high concentrations of DFMO (10 mM) shows no inhibition of accumulation by BSF trypanosomes over the 1 hour time period. This suggests that these two compounds do not share the same mode of uptake. Moreover, when inhibition of tyrosine was performed over a range of DFMO concentrations (up to 5 mM) we found no significant difference in tyrosine uptake. As previously shown tyrosine does inhibit DFMO uptake, however, the experiment was only carried out once. It is possible that with multiple repeats that inhibition seen becomes less significant. Another possibility is the fact that tyrosine itself interacts with a transporter of DFMO uptake without necessarily being a substrate for that carrier.

6.3 Discussion

Amino acid analogues have a great potential for chemotherapy of human African trypanosomiasis. The advantages include the fact that some trypanosome transporters have a high affinity for amino acids and a relatively low abundance of competing substrates. Moreover, amino acid analogues can allow us to assign functional characteristics to transporter systems and allow us to differentiate between a one transporter and multiple-transporter system.

Differences in trypanocidal activity of certain amino acid analogues between PCF and BSF could be as a direct result of uptake or, simply the metabolic differences between parasite stages. The discovery of L-Dopa's trypanocidal action led us to test other relevant compounds (e.g. thyroxine and tri-iodothyronine). These compounds did show some trypanocidal activity and it would be of interest to see if they are targeted through an amino acid transporter. Trypanosome induced hypothyroidism is a phenomenon that has been shown *in vivo* (Lomo *et al.*, 1993). The development of anaemia coincides with the marked decrease in plasma concentration of both tri-iodothyronine and thyroxine, and it seems hormonal treatment enhanced survival of infected animals (Lomo *et al.*, 1995).

As well as multiple systemic features seen in the early acute stage, the late chronic stage of African sleeping sickness is associated with a wide range of neurological features. Neurological features which are common to other neurological disorders associated with monoamine neurotransmitter availability within the brain (Daniel *et al.*, 1976; Kennedy, 2006). These neurotransmitters are synthesized in the brain from aromatic amino acid precursors that have to be obtained from the circulating blood. Changes in the cerebral concentrations of one or more neurotransmitters are the cause of certain neuropsychiatric disorders (Fernstrom & Wurtman, 1971; Shoemaker & Wurtman, 1971; Bernheimer & Hornykiewicz, 1973). The formation of neurotransmitters is largely determined by how the CNS can acquire pre-cursor aromatic amino acids from the circulating blood (Coppen *et al.*, 1963; Daniel *et al.*, 1976). More specifically, elevating brain tyrosine concentrations stimulates catecholamine production (Fernstrom & Fernstrom, 2007).

6.3.1 Azaserine & Glutamine Transport

In this study, the glutamine analogue, azaserine, was found to have differing activities against the two forms of the parasite, with an IC_{50} of 176.46 µM, 10.85 µM, and 1.52 µM against the PCF, BSF, and HEK cell respectively. It could be argued that the toxicity shown by azaserine against the BSF of the parasite (10.85 µM) is moderate at best. For most compounds seeking therapeutic use, nanomolar IC_{50} values are more attractive. There is good evidence that the difference in starting trypanosome densities for these assays does result in a shift in IC_{50} values. This shift could be many orders of magnitude. With that in mind, it is possible that the moderate IC_{50} values shown here by some analogues including azaserine could show higher activities under different conditions.

Previous investigations with other glutamine analogues like DON and acivicin showed that neither is totally active *in vivo*. *T. brucei* infected mice receiving doses of drugs showed no

trypanosomes in the blood (Hofer *et al.*, 2001). However, with discontinuation of therapy trypanosomes reappeared. They concluded that the relapse of trypanosomes after drug removal was observed, suggesting that the glutamine analogues only block the proliferation of the parasites. A functional immune response is then required to eradicate the growth-arrested trypanosomes.

High mammalian cell toxicity rules out any potential therapeutic use, however, the simplicity of the molecule allows chemical remodelling if a drug target can be found. For example, DON is another glutamine analogue similar in structure to azaserine and shows no toxic effects against the mammalian cells used (Appendix 8.14). It would be interesting to test how the small differences between molecules translate to cell toxicity and drug target. Also, even though azaserine is more active against mammalian cells it provides a tool for identification and characterisation of particular amino acid transporters involved in its uptake.

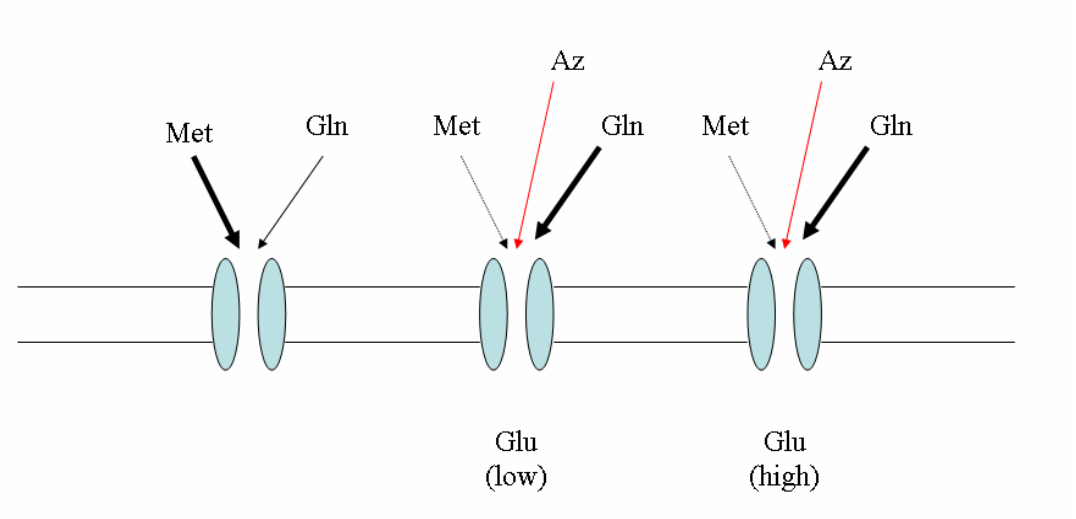


Figure 6-30: Schematic representation of a possible L-glutamine transport system in PCF trypanosomes.

Here, like with other organisms (Kaplan *et al.*, 1959; Brock & Brock, 1960), we have shown azaserine to be transported into the cell by the amino acid transport system, with multiple routes of entry evident (azaserine showed inhibition of glutamine, phenylalanine and tyrosine in both forms of the parasite, although to differing degrees). In the PCF of the parasite we studied this mode of inhibition in more detail, using unlabelled glutamine, glutamate, methionine and azaserine over a range of concentrations (0-10 mM). In each case these amino acids gave a progressive dose-dependent inhibition, with azaserine showing only partial inhibition at the highest concentration used (10 mM). This partial

inhibition exhibited suggests more than one glutamine transporter with azaserine only entering the cell via one transporter. This, coupled to the fact that glutamate inhibition is weak (and at the same time complete) suggests that both glutamine transport systems are inhibited by glutamate but at different concentrations – one at a low glutamate concentration and the other at a high glutamate concentration. Complete inhibition of phenylalanine uptake shows entry via an aromatic amino acid transport system, a transport system which seems not to be shared by other aromatic amino acids.

Previous work investigating the nature of methionine transport in trypanosomes (Hasne & Barrett, 2001) showed a single methionine transporter with high affinity for methionine (showing a K_m value of 30.9 ± 7.6 μM and a V_{max} value of 16.2 ± 0.2 nmol/min/10^8 cells for PCF trypanosomes). Hasne & Barrett (2001) reported the existence in PCF of a single methionine transporter based on the fact that competing amino acids showed weak rather than partial inhibition of methionine uptake. However, competing amino acids (e.g. phenylalanine) do not show any structural similarities to methionine and glutamine inhibition was only 60% at a 5 mM concentration. Here we report an L-glutamine transport system that has high affinity for glutamine (16 μM IC_{50}) and shows weak inhibition by L-methionine (1.39 mM IC_{50}). It is possible that multiple glutamine transporters exist (Figure 6-30), which show differing affinities for glutamate, methionine and azaserine. And that one of the glutamine transporters has a higher affinity for methionine than glutamine. This would be the methionine transporter previously characterised. Uptake of methionine would be poorly inhibited by glutamine as glutamine has another route of uptake. Glutamine uptake is inhibited by methionine, however the other glutamine transporters have a high capacity for glutamine.

L-glutamine and L-tyrosine uptake is evident in BSF trypanosomes, indicating the existence of a similar transport system for these amino acids working in the mammalian stage of the parasite. Moreover, glutamine and tyrosine inhibition by azaserine is still evident. However, it is unknown as to the exact nature of the transport system. It is possible that glutamine internalisation shows a different specificity profile in BSF trypanosomes (different apparent K_i and/or a different inhibiting set of amino acids), which would indicate the expression of a different gene or set of genes specific for this stage of the parasite. This seems likely considering the plethora of amino acid transporter genes found in the genome, some of which show differential expression. Previous work investigating aromatic amino acid uptake in BSF trypanosomes suggested the existence of several aromatic amino acid transporters (M.P. Hasne, thesis). Phenylalanine and

tryptophan were found to use at least 2 routes of entry while a single high affinity transporter was identified for tyrosine

Amino acid recovery of azaserine action is a phenomenon well documented in bacteria (Kaplan *et al.*, 1959; Brock & Brock, 1960) and in *T. equiperdum* (Momparler & Jaffe, 1964). Here no such recovery could be shown by the alamar blue method. This suggests two things; firstly that azaserine's mode of action does not involve amino acid usage or that the effect on amino acid usage is irreversible; and secondly that azaserine penetration through the cell membrane could not be prevented by competing amino acids. Therapeutically, this would be an encouraging feature for a potential drug to have as competing amino acids in the blood plasma or cerebrospinal fluid would have little effect on drug efficacy. The fact that azaserine has multiple routes of entry into the trypanosome interior meant we could not antagonise azaserine action by simple transport competition. The multiple routes of entry also goes some way to explain why resistance through loss of transport could not be easily achieved.

6.3.2 Levodopa & Aromatic Amino Acid Transport

Here we show that Levodopa has toxicity against both forms of the parasite (58.58 µM against PCF and 31.58 µM against BSF). Owolabi *et al* (1989) showed trypanocidal activity of certain catecholamines (dopamine and tryptamine analogues). BSF trypanosomes (10^5 cells/ml) on a feeder layer culture system (*Microtus agrestis* embryonic fibroblasts) incubated with drug (1 µM) were completely cleared after 24 hours. It is possible that the high level of toxicity seen by Owolabi is simply as a result of metabolic activation. *In vivo* tests showed complete clearance of trypanosomes from the bloodstream of mice at a dose of 0.1 mg/kg. The possibility of metabolic activation was explored by testing other compounds of aromatic metabolism in mammalian cells. No other compound showed any significant higher toxicity against trypanosomes. However, an *in vivo* environment is a more complex setting and it is possible that a more complex dynamic between these biologically relevant aromatic analogues interact to bring about an, as yet unknown, trypanocidal effect.

From previous studies (Owolabi *et al.*, 1989), it was not clear if L-Dopa action was due to internal or exterior activity of L-Dopa itself or one or more of its derivatives. L-Dopa entry into the parasite seems to be mediated through an aromatic amino acid transport system, as shown by inhibition of tyrosine uptake. Phenylalanine and tryptophan also inhibited uptake of tyrosine (although to lesser degrees) suggesting a common route of uptake for these

amino acids. However, it seems that more than one transport system is evident. L-Dopa inhibited tyrosine and phenylalanine to different degrees, where if one transport system was apparent, L-Dopa inhibition of phenylalanine would be greater than phenylalanine self-inhibition. The data presented here seems to suggest that L-Dopa entry to the PCF trypanosome is mediated through a tyrosine-specific aromatic amino acid transporter, one which shows some affinity for phenylalanine and tryptophan. In BSF trypanosomes, this inhibition of tyrosine by L-Dopa is still seen, pointing to the existence of a similar transporter at work in that life cycle stage. However, it is unknown as to the exact nature of the transport system. It is possible that glutamine internalisation shows a different specificity profile in BSF trypanosomes (different apparent K_i and/or a different inhibiting set of amino acids), which would indicate the expression of a different gene or set of genes specific for this stage of the parasite.

It is possible that separate transporters exist for the three different aromatic amino acids in PCF trypanosomes. Indeed, separate aromatic amino acid transporters have been postulated in BSF trypanosomes (M.P. Hasne, thesis). With that in mind, it would be wrong to rule out entry of the other aromatic analogues tested just because they showed no significant inhibition of phenylalanine. For example, the analogues 5' hydroxyl-tryptophan and tryptamine, structurally similar to tryptophan are most likely targeted into the cell via a tryptophan specific aromatic amino acid transporter.

L-Dopa toxicity could not be ablated by any of the 20 amino acids in high concentrations, suggesting that a mode of action involving amino acid usage is not at play or, at least, not significant. A possible mode of action involving the creation of reactive oxygen species has been postulated (Owolabi *et al.*, 1989). Detoxification of reactive oxygen species involves the transfer of reducing equivalents to redox cascade pathways (Wilkinson *et al.*, 2003). However, we did not find any increased sensitivity in TbSOD knock-out cell lines. Trypanosome superoxide dismutase enzymes detoxify superoxide anions ($O_2^{\cdot-}$) by catalysing their disproportionation to hydrogen peroxide (McCord & Fridovich, 1969), whereas L-Dopa auto-oxidation produces highly unstable electrophilic Dopa-(semi)quinones and reactive oxygen species such as H_2O_2, O_2^- and OH (Shi *et al.*, 2002). TbSOD would only protect against one class of reactive oxygen species produced by L-Dopa auto-oxidation, which would explain why no difference in sensitivity was observed in mutant TbSOD cell lines.

Growth of trypanosomes in incremental amounts of drug to generate resistant cell lines proved problematic. Progressive darkening of the medium was evident, with turbidity

developing from the production of quinolones and other reactive oxygen species. It seems probable that the trypanocidal activity of L-Dopa is as a result of secondary products of the analogue. Also, we have shown through inhibition of tyrosine uptake that L-Dopa can be transported into cells, suggesting that auto-oxidation can occur both extracellularly and intracellularly. This intracellular and extracellular damage caused by L-Dopa goes some way to explain why a resistance phenotype could not be generated. The non-enzymatic antioxidants L-ascorbic acid, glutathione, N-Acetyl-L-cysteine have been shown to protect mammalian cells *in vitro* against the cytotoxic effects induced by L-Dopa (Lai & Yu, 1997). It would be interesting to see if this protective effect was evident with trypanosomes.

6.3.3 Cell Penetrating Peptides

Cell penetrating peptides as delivery agents for therapeutic compounds are an emerging field. Even with the plethora of recent publications in the field of CPP research, the mechanism for CPP translocation is still poorly understood. The majority of the work on CPPs has been carried out using mammalian cells. However, CPP intracellular accumulation has been observed in a variety of other cell systems, such as yeast (Parenteau *et al.*, 2005), insect cells (Palm *et al.*, 2006), bacteria, fungi (Nekhotiaeva *et al.*, 2004), and tobacco protoplasts (Mae *et al.*, 2005). It is evident from results shown here that the peptide TP10 also has toxic activity against BSF trypanosomes, with trypanocidal activity on cells occurring within 6 hours of drug exposure. CPP entry into the cell is confirmed, yet is highly variable within a given cell population. In some cases, there seems to be some co-localisation with nuclear and kinetoplast intracellular organelles.

Quantum dots are having recent use as luminescent labels for biological systems (la Fuente *et al.*, 2005), mainly because they possess several key advantages over conventional fluorescent dyes: they emit light at a variety of precise wavelengths depending on their size and have long fluorescent lifetimes. In this case, the quantum dot Cadmium sulphide (CdS) was linked to the un-natural amino acid tiopronin which was then functionalised with a Tat protein derived peptide sequence (GRKKRRQRRR). Tiopronin is a pharmaceutically important drug used for the treatment of cystinuria and rheumatoid arthritis (Denneberg *et al.*, 1983).

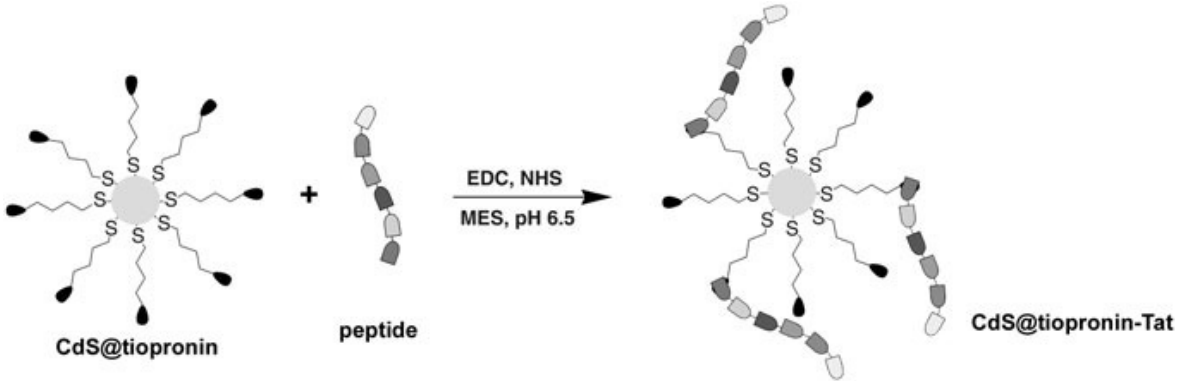


Figure 6-31: Synthesis of CdS-tiopronin-Tat (taken from la Fuente *et al.*, 2005).

Previous studies have shown CdS-tiopronin-QDs coupled to Tat protein-derived peptide sequences were used to achieve nuclear targeting of the nanoparticles in hTERT-BJ1 human fibroblasts (la Fuente *et al.*, 2005). Even after 15 mins incubation fluorescence staining was observed around the cell nucleus, showing evident translocation of the CdS-tiopronin-Tat conjugate to the nucleus. No fluorescence staining was observed when naked CdS-tiopronin was incubated with the cells. Other studies have shown the Tat Peptide to be an efficient molecule to translocate gold nanoparticles into the cell nucleus (la Fuente & Berry, 2005) at a 50 µg/ml concentration after just one hour.

In the case of trypanosomes, along with no trypanocidal activity we have shown there to be no accumulation of the fluorescent conjugate within the cell interior. This suggests that with trypanosomes the tat protein-derived peptide does not have trypanosome cell penetrating properties. With no apparent membrane translocating properties of the tat-derived peptide seen, we can rule out this particular compound as a way to introduce potential trypanocidal drugs into the cell.

6.3.4 Eflornithine

Uptake of 10 µM ^{3}H-DFMO over the 2 hour time period appears to be linear, showing an approximate accumulation of drug at a rate of 10 picomoles per hour (per 10^{7} cells). Uptake in PCF trypanosomes has been previously reported as linear with an accumulation of 2 nanomoles per 10^{9} cells over a 70 min time period, using 0.1 mM DFMO. This is comparable to what is found here in bloodstream forms with 10 pmoles being accumulated over a one hour period using 10 times less DFMO and 100 times fewer cells. With only a single experiment performed in early studies, further work will need to be done to fully complete the preliminary insight gained here. The nature of this apparent linearity of

uptake will need to be explored in more detail. Is the apparent cumulative intake of DFMO as a result of simple diffusion or are transport processes at play?

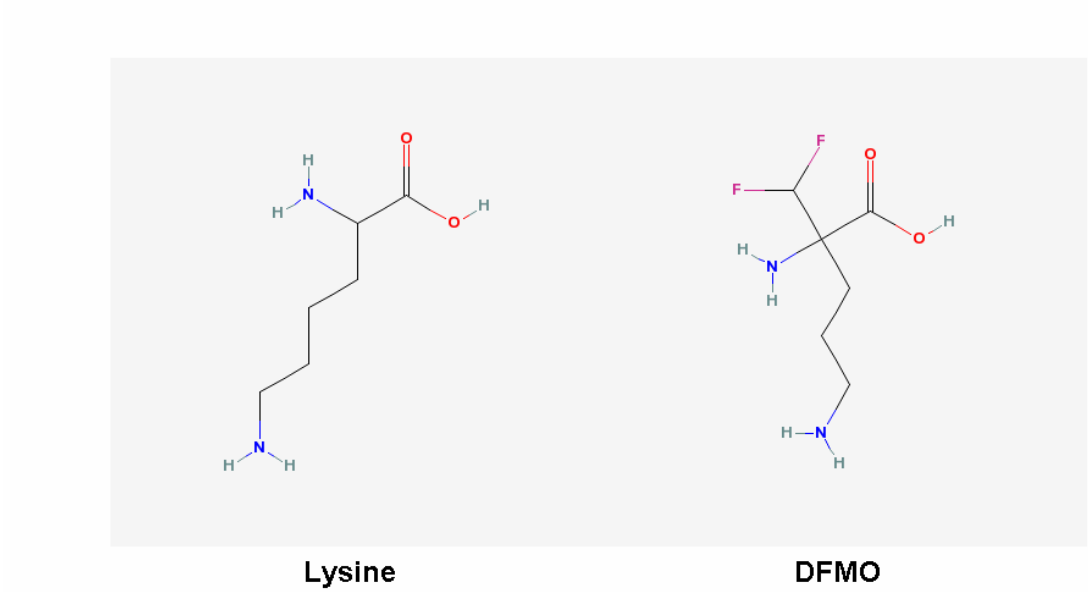


Figure 6-32: Structure of L-lysine and DFMO.

DFMO is structurally similar to the basic amino acid lysine (Figure 6-32), so it is likely that if an active transport component is involved in uptake it would be a route of entry shared with at least one of these amino acids. Lysine differs from DFMO by one CH_2 unit on its hydrophilic side chain and an altered amino acid recognition motif, with the hydrogen attached to the α-carbon replaced by a difluoro-unit. There is evidence to suggest that disruption of the amino acid recognition motif results in abolished uptake of the particular amino acid (Hasne & Barrett, 2001). With methionine transport, the core amino acid structure appears to be essential since analogues consisting of the side chain alone had no inhibitory effect. Fluorine substitution is a powerful tool in bioorganic and medicinal chemistry (Welch & Eswarakrishnan, 1991). Fluorine incorporation into biologically active compounds can alter drug metabolism, enzyme substrate recognition and even affinity for natural receptors (Biffinger *et al.*, 2004). With DFMO the hydrophobic fluoro-carbon group is likely to result in lipid solubility.

In BSF trypanosomes there does possibly seem to be inhibition of DFMO uptake by aromatic amino acids (Figures 6-25, 6-26) although this does not seem to be reciprocal (Figures 6-28, 6-29). DFMO over a range of concentrations showed no inhibition of tyrosine uptake. However, other aromatic amino acids showed more significant inhibition of DFMO uptake (Figure 6-25). Interestingly, when we tried to inhibit DFMO to

completion using all three aromatic amino acids (500 μM concentration each), we still saw significant levels of DFMO uptake. It is possible that for individual aromatic amino acids there is some inhibition exhibited at lower concentrations which is lost at higher ones, either by increased capacity of a transporter or the transient opening of a secondary transporter.

Preliminary studies here have also highlighted the fact that trypanosome amino acid transporters may have the capacity for trans-acceleration or, at least transient regulation. Inhibition of DFMO by aromatic amino acids individually showed some level of inhibition (~80% uptake with tyrosine and ~20% uptake with phenylalanine and tryptophan, Figure 6-26). However, combining all three aromatic amino acids restored DFMO uptake. It is possible that increased concentrations of certain amino acids increases the capacity of a particular transporter of activates another transporter. Trans-acceleration and trans-inhibition phenomenon are well documented in mammalian amino acid transporters (McDowell *et al.*, 1995; Bracy *et al.*, 1986; Fong *et al.*, 1990; Volk *et al.*, 2003; Pan *et al.*, 2002).

More significantly, lysine uptake shows significant inhibition by increasing concentrations of DFMO (Figure 6-27) as well as a dose dependent self inhibition, giving an apparent IC_{50} of 39 μM ± 4. From this, it seems evident that some transport-mediated process exists for the internalisation of the drug, possibly through a lysine specific transporter.

Inhibition of DFMO uptake using amino acids has previously been looked at (Bitonti *et al.*, 1985). However, due to the methodology of the protocol no apparent inhibition was seen. DFMO inhibition was only studied at a single 60 min time point, a time point which is far too long to report exclusively upon transport processes, especially when a diffusion component could also be at play. Also, inhibition studies only used concentrations barely 20 times that of DFMO (56 μM DFMO vs 1 mM analogue). Here, a number of amino acids have been shown to inhibit DFMO uptake to a significant extent. The fact that cold DFMO only inhibits [^{3}H]-DFMO to a maximum of 50%, even in 500-fold excess, suggests that if a transport system is involved then affinity for DFMO is weak at best. It is possible the accumulation of DFMO observed here and with other studies is the net effect of transport and diffusion.

What needs to be done now is to try and separate the components involved in the internalisation of DFMO as well as trying to decipher the specifics of the amino acid transport system involved. There is the aforementioned active transport versus simple

diffusion debate as well as an apparent stimulation of uptake at higher concentrations. Transport versus diffusion can be easily separated. Uptake studies at 4ºC would help eliminate the transport component. Also, with regards to BSF aromatic amino acid transporters, further investigation of individual uptake is necessary (tyrosine, phenylalanine, and tryptophan). Multiple aromatic amino acid transporters have already been postulated (M.P. Hasne, thesis). What needs to be determined if these transport systems have the capability of transinhibition, or what seems like a loss of inhibition at high concentrations of more than one inhibitor.

It has often been mentioned that it is possible that the contribution of an active transport system to the therapeutic effects of DFMO would be insignificant (Bitonti *et al.*, 1985). But in studies with resistant cell lines only uptake seems implicated in the resistance phenotype (Phillips & Wang, 1987). It is entirely possible that a simple increase in ornithine uptake is enough to compensate for the effects of DFMO, as it has been seen that ornithine uptake is significantly faster in resistant cell lines as well as wild type cells exposed to DFMO. The resistance phenotype could simply be a result of the establishment of sustained increase of ornithine uptake, due to increase in transporter activity. This, coupled with increase in polyamine and arginine intake, could lead to the apparent resistance phenotype seen.

7 Closing Discussion

The long-held beliefs on neglected disease drug development activity are no longer accurate (Moran, 2005). Neglected diseases, once thought of by pharmaceutical companies as non-profitable, are finding more research activity. This reflects the formation of more and more new pharmaceutical industry neglected-disease institutes, the creation of new drug development Public-private partnerships (PPPs), which now conduct three-quarters of all identified neglected disease drug development (Moran, 2005).

There is a shifting landscape in drug discovery against neglected diseases. Safer and more affordable drugs are still needed for the tropical diseases of the developing world. Only 13 new drugs have been developed for neglected tropical diseases since 1975 (Moran, 2005), and many have proved to be too expensive, too toxic or too difficult to administer in resource poor settings. While older front-line drugs are losing their efficacy due to the emergence of widespread drug resistance. To this effect, it is important to generate validated and druggable targets of the African trypanosome. The genome has opened up a vast library from which researchers can start from. Transporters themselves do not necessarily offer much of a viable drug targets, but it has been shown that transporters prove a viable gateway for potential trypanocides. The role of the P2 nucleoside transporter as a transporter for trypanocidal drugs and its role in resistance mechanisms has set precedents for features of transporters that make potential drug carriers. Amino acid transporters share the same characteristics (Hasne & Barrett, 2000).

A family of 46 genes encoding amino acid transporters is present in the *T. brucei* genome making it the largest, and most diverse, family of transporters encoded in the trypanosome genome. It was one aim of this project to evaluate amino acid transporters as a mode of entry for potential trypanocidal drugs. Many compounds that have been given some attention as potential trypanocides (azaserine and eflornithine) do show targeting through amino acid transporters. Characterisation of amino acid transporters by molecular biology and biochemical techniques was performed allowing evaluation of targeting of amino acid analogues with trypanocidal activity through amino acid transporters.

Targeting amino acid-like compounds through amino acid transporters proves a viable method for introducing trypanocidal compounds into the cell interior. In the mammalian system, there is potential for amino acid transporters as delivery systems for drugs and prodrugs. Recent studies have shown the potential of the human amino acid transporter $ATB^{0,+}$, a transporter of neutral as well as cationic amino acids, as a drug delivery system (Ganapathy & Ganapathy, 2005). Certain antiviral drugs can be transported by the transporter when linked to the side chain of anionic amino acids.

Moreover, because the brain capillary endothelial cells comprising the BBB greatly prohibits transport of certain solutes from the blood, due to tight junctions, high brain capillary metabolism, low pinocytic vesicular trafficking, and efficient efflux mechanisms (Killian *et al.*, 2007) it is important to utilise carrier-mediated transport processes into the brain. Amino acid analogues offer an attractive class of compounds that can take advantage of brain amino acid transporters for late stage sleeping sickness.

With recent studies showing that a strategy comprising of covalent coupling of small molecules to L-cysteine achieves high affinity recognition for the specific, cerebrovascular large neutral amino acid transporter (LAT1) (Killian *et al.*, 2007), it may be possible to attach an amino acid recognition motif to other potential trypanocides that might allow targeting through either mammalian or trypanosome amino acid transporters.

One amino acid transporter, TbAATP1, was characterised. It was shown to be a carrier of small, neutral amino acids. Its substrate recognition profile will allow drug design of potential trypanocides that can be targeted through TbAATP1. Moreover, modes of resistance involving loss of the transporter can be investigated with the use of the RNAi cell line.

Grouping of amino acid transporter genes based on gene sequence (rather than chromosomal location) aides down-stream functional genomic approaches to characterisation. The fact that most genes that group together by sequence usually tend to group together by genomic locus means that gene knock-out approaches offer an advantage over conventional RNAi based approaches. However, with some large arrays, sometimes stretching to more than 15 kb in size, means that large gene families will prove difficult to knock-out by homologous recombination. The limitations of RNAi means it will prove difficult to distinguish between most of the amino acid transporter genes in terms of functional significance. A further complication is functional redundancy in amino acid transporter repertoire. Further investigations into amino acid transporters needs to be a multi-faceted approach, with biochemical and gene knock-out techniques employed to allow assignment of functional phenotype to gene.

Inhibition profiles play a major part of characterising transporters. It is important that the inhibition characteristics of transporters either with individual amino acids or multiple amino acids. This will allow predictions of *in vivo* settings and bioavailability within the trypanosome in the presence of changing extracellular amino acid concentrations (amino acid concentrations in the blood and/or cerebrospinal fluid as a function of disease state).

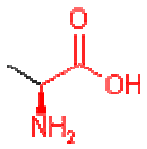
Differential regulation throughout the life cycle of amino acid transporters also needs to be investigated in more detail. This will not only allow functional redundancy of genes to be filtered out, but also highlight transporters that become essential in the mammalian host. Allowing, trypanocidal amino acid analogue to show greater efficacy. Moreover, differential regulation of amino acid transporters modulating uptake of trypanocides might result in changes in susceptibility.

Characterising amino acid transporters offers a window into amino acid metabolism in these parasites. L-proline utilisation has been shown to be regulated by overrall glucose availability. D-glucose exerts an influence on other aspects of procyclic trypanosome biology, including procyclin expression as well as mitochondrial energy generation. It is possible that glucose usage has implications for tsetse fly biology and could be linked to developmental cues of the parasite.

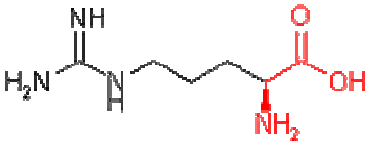
Our knowledge of the parasite and parsite-host interactions are forever evolving, aided by new tools and new collaborative efforts. Systems biology is such a field that brings together a multi-disciplinary approach to investigate the biochemistry of the parasite. Metabolomics offers a powerful technique to un-biasly follow the fate of metabolites within cells exposed to different conditions. Moreover, pertubations and adaptational processes can be seen. The greatest potential metabolomics holds for trypanosome biology is to build upon genome sequencing data. With only half of the genome annotated means the actual metabolome of African trypanosome is much larger than the *in silico* prediction. The field of metabolomics is in the early stages of development and it is important to follow up the changes seen with biochemical characterisation. Only then can metabolomics live up to its full potential.

8 Appendix

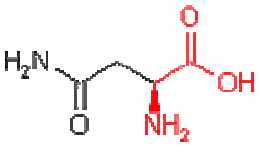
8.1 Amino Acids



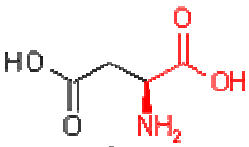
Ala



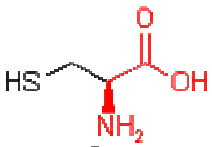
Arg



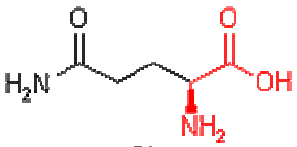
Asn



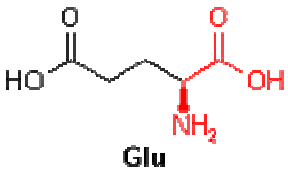
Asp



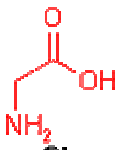
Cys



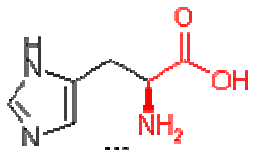
Gln



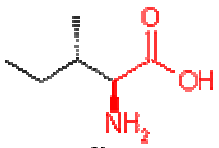
Glu



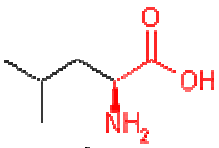
Gly



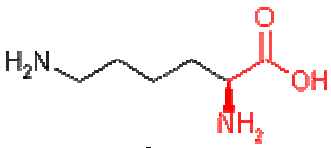
His



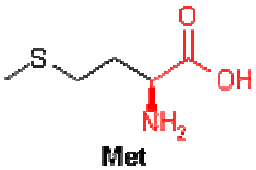
Ile



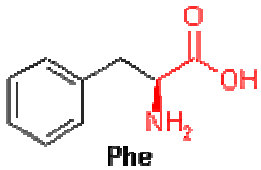
Leu



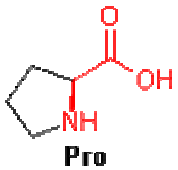
Lys



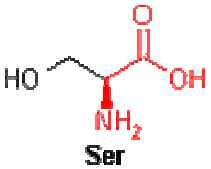
Met



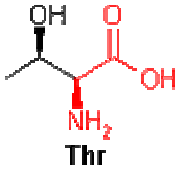
Phe



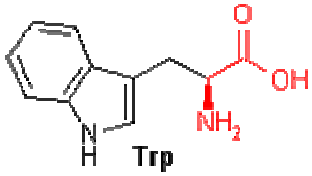
Pro



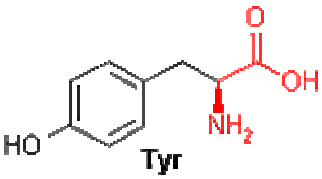
Ser



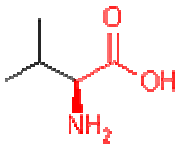
Thr



Trp



Tyr



Val

8.2 SDM80

NaH_2PO4	157 mg
NaCl	6.8 g
$MgSO_4$	100 mg
KCl	400 mg
$CaCl_2$	200 mg
L-Arginine	100 mg
L-Methionine	70 mg
L-Phenylalanine	80 mg
L-Threonine	350 mg
L-Tyrosine	100 mg
Taurine	160 mg
L-Alanine	200 mg
L-Asparagine	13.2 mg
L-Aspartate	13.3 mg
L-Glutamate	14.7 mg
L-Glutamine	200 mg
Glycine	7.5 mg
L-Serine	60 mg
HEPES	8 g
MOPS	5 g
$NaHCO_3$	2.2 g
Pyruvate	220 mg
Mercaptoethanol (0.1 M)	2 ml
Hypoxanthine	14 mg
Thymidine	4 mg
Vitamins (100 X)	10 ml
Essential amino acids (50 X)	20 ml
Phenol Red	4 ml
Hemin (2.5 mg/ml)	2 ml
Dialysed Foetal Calf Serum	10%

For 1 litre. Filter sterilise. Store at 4ºC.

8.3 CBSS Buffer

HEPES	25 mM
$NaCl_2$	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

For 1 litre. Adjust to pH 7.4 and store at –20 °C.

8.4 Cunningham's Medium

$CaCl_2$	113 mg
KCl	3000 mg
$MgSO_4$	1800 mg
$NaH_2PO_4.H_2O$	530 mg
Glucose	700 mg
Fructose	400 mg
Sucrose	400 mg
Phenol Red	21 mg
HEPES	6000 mg
Alanine	550 mg
Beta alanine	2000 mg
Arg.HCl	440 mg
Asparagine	240 mg
Aspartic acid	110 mg
Cystine	110 mg
Glutamic acid	250 mg
Glutamine	1700 mg
Glycine	120 mg
$Histidine.HCl.H_2O$	160 mg
Isoleucine	90 mg
Leucine	90 mg
Lysine.HCl	187 mg
Methionine	100 mg
Phenylalanine	200 mg
Proline	6900 mg
Serine	100 mg
Taurine	270 mg
Threonine	50 mg
Tryptophan	100 mg
Tyrosine	200 mg
Valine	100 mg
Pyruvate	100 mg
Hypoxanthine	116 mg
FCS	20%

For 1 litre. Filter sterilise and store at 4ºC.

8.5 TELT Buffer

Tris-HCl (pH 8)	50 mM
EDTA (pH 9)	62.5 mM
LiCl	2.5 M
Triton X-100	4% v/v

8.6 TE Buffer

Tris-HCl (pH 8)	10 mM
EDTA (pH 8)	1 mM

8.7 1X TAE Buffer

Tris acetate (pH 8.5)	40 mM
EDTA	1 mM

8.8 LB Medium

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

For 1 litre. Sterilise by autoclaving.

8.9 20X SSC

Tri-sodium citrate	88.23 g
NaCl	175.32 g

For 1 litre. Adjust to pH 7. Store at room temperature.

8.10 10X MOPS

MOPS	0.2 M
Sodium acetate	80 mM
EDTA (pH 7)	10 mM

Filter and store in the dark at 4ºC.

8.11 ZPFM Buffer

NaCl	132 mM
KCl	8 mM
$Na_2H_2PO_3^{2+}$	8 mM
KH_2PO_3	1.5 mM
$Mg(C_2H_3O_2)$	1.5 mM
$C_4H_6CaO_4$	90 µM

8.12 PBS

Sodium phosphate	20 mM
NaCl	150 mM

8.13 100X Denhardt's Solution

Bovine serum albumin	2 g
Ficoll	400.2 g
Polyvinylpyrolidone	2 g

For 100 ml. Store at -20ºC.

8.14 Amino Acid Analogues

Analogue	PCF	BSF	HEK
Azaserine	176.46	10.85	1.52
Glycine Hydroxamate	N/A	N/A	N/A
L-Serine b-naphthylamide	38.60	43.70	179.60
Serine Hydroxamate	N/A	N/A	N/A
Serine Benzyl Ester	N/A	N/A	N/A
Serine Methyl Ester	N/A	N/A	N/A
N-acetyl D, L-serine	N/A	N/A	N/A
Arginine Methyl Ester	N/A	N/A	N/A
Acetyl Methionine	N/A	N/A	N/A
N-formyl Methionine	N/A	N/A	N/A
Alanine Ethyl Ester	N/A	N/A	N/A
Seleno Methionine	N/A	N/A	N/A
Lysine Methyl Ester	N/A	N/A	N/A
Alanyl Glycine	N/A	N/A	N/A
Adenosyl Homocysteine	N/A	N/A	N/A
Seleno Ethionine	N/A	N/A	N/A
Tyrosine Methyl Ester	N/A	N/A	N/A

N-Acetyl Tyrosine Ethyl Ester	N/A	N/A	N/A
Tryptophan Benzyl Ester	79.21	27.54	N/A
Fluoro Tryptophan	N/A	N/A	N/A
Kynurenine	N/A	N/A	N/A
Saccharopine	N/A	N/A	N/A
Tryptophan Ethyl Ester	28.00	N/D	N/D
N-formyl Phenylalanine	N/A	N/A	N/A
N3 Trimethyl Lysine	N/A	N/A	N/A
N-formyl Tyrosine	N/A	N/A	N/A
N-Acetyl Tyrosineamide	N/A	N/A	N/A
N-Acetyl Tryptophanamide	N/A	N/A	N/A
AS10	N/A	N/A	N/A
CMC-20-1	N/A	N/A	N/A
AJ222	N/A	N/A	N/A
AS65	N/A	N/A	N/A
KF38	N/A	N/A	N/A
CMC-18-2	N/A	N/A	N/A
AS66	N/A	N/A	N/A
L-Alaninamide	N/A	N/A	N/A
O-Phospho-L-Serine	N/A	N/A	N/A
L-3,4-Dihydroxy Phenylalanine (L-DOPA)	58.60	31.58	112.21
DL-Threo-B-Phenylserine	N/A	N/A	N/A
L-Homocysteine Thiolactone	N/A	N/A	N/A
Cycloserine	141.80	327.85	176.42
N-Succinyl-L-Phenylalanine p-nitroanilide	N/A	N/A	N/A
Hippuryl-L-Arginine	N/A	N/A	N/A
Diazoacetyle-dl-Norleucine Methyl Ester	N/A	N/A	N/A
N-Benzoyl-L-Propyl-L-Phenyalanyl-L-Arginine p-nitroanilide	N/A	N/A	N/A
N-Benzoyl-L-Propyl-L-Tyrosine p-nitroanilide	46.21	23.34	110.00
L-Propyl Glycine	N/A	N/A	N/A
S-Benzyl L-Cysteine p-nitroanilide	N/A	N/A	N/A
L-Phenylalanyl L-Proline	N/A	N/A	N/A
a-N-Benzoyl L-Arginine Amide	N/A	N/A	N/A
Benzoyl L-Valyl-Glycyl L-Arginine p-Nitroanilide	N/A	N/A	N/A
N-Benzoyl L-Tyrosine Ethyl Ester	N/A	N/A	N/A
Na-Acetyl-Glycyl-L-Lysine B-Naphthyl Ester	48.44	N/A	N/D
Glycyl-L-Leucine	N/A	N/A	N/A
CycloLeucine	N/A	N/A	N/A
N-(p-Aminobenzoyl)-L-Glutamic Acid	N/A	N/A	N/A
Glycine Benzyl Ester	N/A	N/A	N/A
L-Leucine B-Naphthyl Amide	69.66	40.60	N/D
DL-B-Hydroxy Norvaline	N/A	N/A	N/A
Dihydrofolic Acid	N/A	N/A	N/A
DL-Tetrahydrofolic Acid	N/A	N/A	N/A
3,4 Dihydrophenylacetic acid	65.20	390.59	112.40
5' Hydroxy-L-Tryptophan	N/A	N/A	N/A
Serotonin	155.00	N/A	N/A
Tryptamine	21.90	57.83	176.42
Dopamine	63.25	275.05	87.07
N-Acetyl DL Tryptophan	N/A	N/A	N/A
DL-a-Amino Adipic Acid	N/A	N/A	N/A
m-Flouro-DL-Tyrosine	N/A	N/A	N/A
5-Flouro-DL-Tryptophan	N/A	N/A	N/A
5-methly DL Tryptophan	N/A	N/A	N/A

DL-p-Hydroxyphenyl-Lactic Acid	N/A	N/A	N/A
5-Hydroxy L Tryptophan	N/A	N/A	N/A
DL Indole-3-Lactic Acid	N/A	N/A	N/A
Cyclophosamide	N/A	N/A	N/A
L-Canavanine	N/A	N/A	N/A
D-y-MethylLeucine	N/A	N/A	N/A
O-Benzyl-L-Tyrosine	N/A	N/A	N/A
Tri-Iodothyronine	134.44	102.17	N/A
L-Thyroxine	N/A	56.17	N/A
Melphalan	N/A	102.23	N/A
Azetidine-2-Carboxylic Acid	N/A	N/A	N/A
NorLeucine	N/A	N/A	N/A
Methionine Sulfoximine	N/A	N/A	N/A
Hydroxy Lysine	N/A	N/A	N/A
Noradrenaline	N/A	N/A	27.90
Ureidosuccinic acid-N-carbomyl Aspartate	N/A	N/A	N/A
DON	N/D	N/D	N/A

8.15 List of Metabolites

Metabolite	Mass	Formula
Acetaldehyde	44.026215	C2H4O
Acetate	60.02113	C2H4O2
Ethanolamine	61.052763	C2H7NO
Pyruvaldehyde	72.02113	C3H4O2
Glycine	75.03202841	C2H5NO2
Pyruvate	87.00822	C3H3O3-
Pyruvic Acid	88.016045	C3H4O3
Putrescine	88.100048	C4H12N2
Alanine	89.04767847	C3H7NO2
sarcosine	89.04767847	C3H7NO2
Dihydroxyacetone	90.03169406	C3H6O3
glycerol	92.04734412	C3H8O3
Ethanolamine HCl	97.02944159	C2H8ClNO
L-a-Amino-n-butyric acid	103.0633285	C4H9NO2
Serine	105.0425931	C3H7NO3
D1-Pyrroline-5-carboxylic acid	113.0476785	C5H7NO2
proline	115.0633285	C5H9NO2
Succinate	116.01096	C4H4O4
Amino oxobutyrate	117.042594	C4H7NO3
valine	117.0789786	C5H11NO2
Threonine	119.0582432	C4H9NO3
L-homoserine	119.058244	C4H9NO3
Cysteine	121.0197492	C3H7NO2S
Taurine	125.0146638	C2H7NO3S
Pyro-L-glutamic acid	129.0425931	C5H7NO3
L-Pipecolic acid	129.0789786	C6H11NO2
Hydroxyproline	131.0582432	C5H9NO3
Glutamate γ-semialdehyde	131.058244	C5H9NO3
Leucine	131.0946287	C6H13NO2

Malate	132.005875	C4H4O5
Oxaloacetate	132.005875	C4H4O5
Asparagine	132.0534921	C4H8N2O3
Glycyl-glycine	132.0534921	C4H8N2O3
Ornithine	132.089878	C5H12N2O2
Aspartic acid	133.0375077	C4H7NO4
L-Homocysteine	135.0353992	C4H9NO2S
Adenine	135.0544952	C5H5N5
Phosphoethanolamine	141.0190943	C2H8NO4P
Spermidine	145.157897	C7H19N3
2-ketoglutarate	146.021525	C5H6O5
L-Glutamine	146.0691422	C5H10N2O3
Lysine	146.1055277	C6H14N2O2
L-Glutamic acid	147.053159	C5H9NO4
O-acetyl-L-serine	147.053159	C5H9NO4
Mevalonic acid	148.07356	C6H12O4
Methionine	149.0510493	C5H11NO2S
Histidine	155.0694765	C6H9N3O2
Orotic acid	156.0171066	C5H4N2O4
L-a-Aminoadipic acid	161.0688078	C6H11NO4
O-acetyl-L-homoserine	161.068809	C6H11NO4
Carnitine	161.1051934	C7H15NO3
Hydroxylysine	162.1004423	C6H14N2O3
N-Acetyl-L-cysteine	163.0303139	C5H9NO3S
L-Methionine sulfoxide	165.0459639	C5H11NO3S
Phenylalanine	165.0789786	C9H11NO2
Phosphoenolpyruvate	167.982378	C3H5O6P
L-Cysteic acid	169.004493	C3H7NO5S
N3-Methyl-L-histidine	169.0851266	C7H11N3O2
Dihydroxyacetone phosphate	169.9980245	C3H7O6P
Glyceraldehyde 3 phosphate	169.998028	C3H7O6P
Glycerol 3 phosphate	172.013678	C3H9O6P
Glycyl-L-proline	172.0847923	C7H12N2O3
N-Acetyl-L-hydroxyproline	173.0688078	C7H11NO4
N-Acetyl-L-ornithine	174.1004423	C7H14N2O3
Arginine	174.111676	C6H14N4O2
L-Citrulline	175.0956913	C6H13N3O3
Glucose	180.0633881	C6H12O6
L-Methionine sulfone	181.0408785	C5H11NO4S
Tyrosine	181.0738932	C9H11NO3
DFMO	182.086684	C6H12F2N2O2
L-Homocysteic acid	183.0201431	C4H9NO5S
Glycerate 3-phosphate	185.9929391	C3H7O7P
NE-Acetyl-L-lysine	188.1160924	C8H16N2O3
L-Homocitrulline	189.1113414	C7H15N3O3
gamma-Carboxyglutamate	191.042987	C6H9NO6
Citrate	192.027005	C6H8O7
L-3-(3,4-Dihydroxyphenyl)-alanine	197.0688078	C9H11NO4
erythrose-4-phosphate	200.008593	C4H9O7P
S-Sulfo-L-cysteine	200.9765637	C3H7NO5S2
NG,NG-Dimethylarginine	202.1429758	C8H18N4O2
Spermine	202.215746	C10H26N4
Tryptophan	204.0898776	C11H12N2O2
L-Kynurenine	208.0847923	C10H12N2O3

1-deoxy-D-xylulose 5-phosphate	214.024242	C5H11O7P
2-C-methyl-D-erythritol 4-phosphate	216.039892	C5H13O7P
O-succinyl-L-homoserine	219.074289	C8H13NO6
5-Hydroxy-L-tryptophan	220.0847923	C11H12N2O3
L-Cystathionine	222.0674276	C7H14N2O4S
cystathionine	222.06743	C7H14N2O4S
3-Hydroxy-L-kynurenine	224.0797069	C10H12N2O4
Carnosine	226.1065903	C9H14N4O3
phosphatidic acid	228.0035038	C5H9O8P
Mevalonate-5-phosphate	228.039892	C6H13O7P
Ribose-5-phosphate	230.019158	C5H11O8P
Ribulose-5-phosphate	230.019158	C5H11O8P
Xyulose-5-phosphate	230.019158	C5H11O8P
L-Cystine	240.0238483	C6H12N2O4S2
Anserine	240.1222404	C10H16N4O3
Isopentenyl diphosphate	246.005831	C5H12O7P2
Dimethylallyl diphosphate (DMAPP)	246.005831	C5H12O7P2
gamma-glutamylcysteine	250.062345	C8H14N2O5S
L-Cysteine-L-homocysteine	254.0394983	C7H14N2O4S2
palmitic acid	256.2402303	C16H32O2
6-phosphogluconolactone	258.014073	C6H11O9P
Fructose-6-phosphate	260.029723	C6H13O9P
Glucose-6-phosphate	260.029723	C6H13O9P
HMBDP	262.000746	C5H12O8P2
1,3-Bisphosphoglycerate	265.9592695	C3H8O10P2
Adenosine	267.096755	C10H13N5O4
L-Homocystine	268.0551484	C8H16N2O4S2
Inosine	268.0807695	C10H12N4O5
6-phosphogluconate	276.024638	C6H13O10P
L-Saccharopine	276.1321364	C11H20N2O6
MEcPP	277.995661	C5H12O9P2
linolenic acid	278.2245802	C18H30O2
linoleic acid	280.2402303	C18H32O2
Oleic acid	282.2558803	C18H34O2
Melanostatin	284.1848407	C13H24N4O3
Stearic acid	284.2715304	C18H36O2
Orotidine	288.0593654	C10H12N2O8
Sedoheptulose-7-phosphate	290.040288	C7H15O10P
L-Argininosuccinic acid	290.1226343	C10H18N4O6
Aspartame	294.1215717	C14H18N2O5
Methylthioadenosine (MTA)	297.089562	C11H15N5O3S
lysophosphatidylcholine	299.1133886	C10H22NO7P
arachidonic acid	304.2402303	C20H32O2
Glutathion (GSH)	306.099793	C10H18N4O5S
glutathione	307.083806	C10H17N3O6S
Mevalonate-5-pyrophosphate	308.006226	C6H14O10P2
gondoic acid	310.2871805	C20H38O2
Glorin	327.1794209	C15H25N3O5
Docosahexaenoic acid	328.2402303	C22H32O2
beta-Aspartylglucosamine	335.1328647	C12H21N3O8
Kyotorphin	337.1750043	C15H23N5O4
erucic acid	338.3184806	C22H42O2
Fructose 1,6 bisphosphate	339.996056	C6H14O12P2
AMP	347.063086	C10H14N5O7P

d S-adenosylmethionine (dSAM)	354.147411	C14H22N6O3S
S-Adenosyl-L-homocysteine	384.1215885	C14H20N6O5S
phosphatidylserine	385.1137825	C13H24NO10P
cholesterol	386.3548661	C27H46O
Ergosterol	396.339214	C28H44O
S-adenosylmethionine (SAM)	398.1372385	C15H22N6O5S
acylcarnitine	399.1450636	C15H23N6O5S+
S-adenosylmethionine	415.139981	C15H23N6O6S
ADP	427.0294148	C10H15N5O10P2
glutathionylspermidine	434.231141	C17H34N6O5S
phosphatidyl glycerol	455.2046089	C19H36O10P-
ATP (Oxidised)	504.980104	C10H14N5O13P3
ATP	506.9957452	C10H16N5O13P3
CDP-ME	521.081183	C14H25N3O14P2
CDP-MEP	601.047516	C14H26N3O17P3
Glutathione	612.1519619	C20H32N6O12S2
sphingomyelin	647.5127996	C35H72N2O6P+
NAD	663.1091216	C21H27N7O14P2
NAD (oxidised)	664.116954	C21H28N7O14P2+
phosphatidylglycerol	670.405714	C32H63O12P
Trypanothione (oxidised)	721.288735	C27H47N9O10S2
Trypanothione	722.2965552	C27H48N9O10S2
Trypanothione (reduced)	723.304384	C27H49N9O10S2
NADP	743.075458	C21H28N7O17P3
NADP (oxidised)	744.0832771	C21H29N7O17P3+
NADPH	745.0911021	C21H30N7O17P3
phosphatidylethanolamine	748.5856302	C41H83NO8P+
phosphatidylcholine	759.5778051	C42H82NO8P
Acetyl-CoA	809.1257731	C23H38N7O17P3S
Acetoacetyl-CoA	851.136349	C25H40N7O18P3S
succinyl-CoA	867.131264	C25H40N7O19P3S
HMG-CoA	911.157479	C27H44N7O20P3S
phosphatidylinositol	1046.48979	C47H85O19P3
cardiolipin	1150.800075	C58H120O17P2
Galactose	180.06339	C6H12O6
D-Galactono-1,4-lactone	178.04774	C6H10O6
D-Galactonate	196.058305	C6H12O7
2-Dehydro-3-deoxy-D-galactonate	178.04774	C6H10O6
2-DD-galactonate 6-phosphate	258.014073	C6H11O9P

8.16 Biochemical Transformations

Alanine	C3H5NO	71.037113835
Arginine	C6H12N4O	156.101111124
Asparagine	C4H6N2O2	114.042927522
Aspartic Acid	C4H5NO3	115.026943115
Cysteine	C3H5NOS	103.009185635
Cystine	C6H10N2O3S2	222.01328591
Glutamic Acid	C5H7NO3	129.042593189
Glutamine	C5H8N2O2	128.058577596

Glycine	C2H3NO	57.021463761
Histidine	C6H7N3O	137.058911929
Isoleucine	C6H11NO	113.084064057
Leucine	C6H11NO	113.084064057
Lysine	C6H12N2O	128.094963104
Methionine	C5H9NOS	131.040485783
Phenylalanine	C9H9NO	147.068413983
Proline	C5H7NO	97.052763909
Serine	C3H5NO2	87.032028475
Threonine	C4H7NO2	101.047678549
Tryptophan	C11H10N2O	186.07931303
Tyrosine	C9H9NO2	163.063328623
Valine	C5H9NO	99.068413983
acetotacetate (-H2O)	C4H4O2	84.021129428
acetone (-H)	C3H5O	57.034039825
adenylate (-H2O)	C10H12N5O6P	329.052521734
biotinyl (-H)	C10H15N2O3S	243.080339295
biotinyl (-H2O)	C10H14N2O2S	226.077599618
carbamoyl P transfer (-H2PO4)	CH2ON	44.013638724
co-enzyme A (-H)	C21H34N7O16P3S	765.099565568
co-enzyme A (-H2O)	C21H33N7O15P3S	748.096825891
glutathione (-H2O)	C10H15N3O5S	289.073242585
isoprene addition (-H)	C5H7	67.054775259
malonyl group (-H2O)	C3H2O3	86.000393994
palmitoylation (-H2O)	C16H30O	238.22966575
pyridoxal phosphate (-H2O)	C8H8NO5P	229.014010906
urea addition (-H)	CH3N2O	59.024537771
adenine (-H)	C5H4N5	134.046670198
adenosine (-H2O)	C10H11N5O3	249.086189377
Adenosine 5'-diphosphate (-H2O)	C10H13N5O9P2	409.018854091
Adenosine 5'monophosphate (-H2O)	C10H12N5O6P	329.052521734
cytidine 5' diphosphate (-H2O)	C9H13N3O10P2	385.007620711
cytidine 5' monophsophate (-H2O)	C9H12N3O7P	305.041288354
cytosine (-H)	C4H4N3O	110.035436818
Guanosine 5- diphosphate (-H2O)	C10H13N5O10P2	425.013768731
Guanosine 5- monophosphate (-H2O)	C10H12N5O7P	345.047436374
guanine (-H)	C5H4N5O	150.041584838
guanosine (-H2O)	C10H11N5O4	265.081104017
deoxythymidine 5' diphosphate (-H2O)	C10H14N2O10P2	384.012371738
thymidine (-H2O)	C10H12N2O4	224.079707024
thymine (-H)	C5H5N2O2	125.035102485
thymidine 5' monophosphate (-H2O)	C10H13N2O7P	304.046039381
uridine 5' diphosphate (-H2O)	C9H12N2O11P2	385.991636304
uridine 5' monophosphate (-H2O)	C9H11N2O8P	306.025303947
uracil (-H)	C4H3N2O2	111.019452411
uridine (-H2O)	C9H10N2O5	226.05897159
acetylation (-H)	C2H3O2	59.013304391
acetylation (-H2O)	C2H2O	42.010564714
C2H2	C2H2	26.015650074
Carboxylation	CO2	43.98982928
CHO2	CHO2	44.997654317
condensation/dehydration	H2O	18.010564714
diphosphate	H3O6P2	160.940489751
ethyl addition (-H2O)	C2H4	28.031300148

Formic Acid (-H2O)	CO	27.99491464
glyoxylate (-H2O)	C2O2	55.98982928
hydrogenation/dehydrogenation	H2	2.015650074
hydroxylation (-H)	O	15.99491464
Inorganic Phosphate	P	30.9737634
ketol group (-H2O)	C2H2O	42.010564714
methanol (-H2O)	CH2	14.015650074
phosphate	HPO3	79.966332357
primary amine	NH2	16.018724084
pyrophosphate	PP	61.9475268
secondary amine	NH	15.010899047
sulfate (-H2O)	SO3	79.95681572
tertiary amine	N	14.00307401
C6H10O5	C6H10O5	162.05282357
C6H10O6	C6H10O6	178.04773821
D-Ribose (-H2O) (ribosylation)	C5H8O4	132.042258856
disaccharide (-H2O)	C12H20O11	340.10056178
glucose-N-Phosphate (-H2O)	C6H11O8P	242.019155927
Glucuronic Acid (-H2O)	C6H8O6	176.032088136
monosaccharide (-H2O)	C6H10O5	162.05282357
trisaccharide (-H2O)	C18H30O15	486.15847071

Glossary

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“ma voix est faible…”

“…et meme, un peu profane.”