A COMPARATIVE STUDY OF PURINE NUCLEOBASE AND NUCLEOSIDE TRANSPORTERS IN PROTOZOAN SPECIES

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DECLARATION

I declare that the results presented in this thesis are my own work and that, to the best of my knowledge, it contains no material previously substantially overlapping with material submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

Mohammed I. Al-Salabi

ABSTRACT

Purine nucleobase and nucleoside transporters play an important role in the physiology and biochemistry of protozoan parasites, because these parasites can not synthesize purines *de novo* and depend upon salvaging the purines from their hosts. The differences in purine metabolism between these parasites and their mammalian host could be of great pharmacological importance to the rational design of new drugs. In addition, purine transporters are interesting to the current pharmacology of these important human pathogens, as they have been shown to mediate the uptake of purine analogues such as allopurinol and several front-line non-purine drugs that are selectively cytotoxic to the parasites. Selective delivery of drugs to parasites via plasma membrane transporters offers an effective approach to specifically target pathogens. Using biochemical techniques, we have identified and characterised a number of purine nucleobase and nucleoside transporters in protozoan parasites such as *Leishmania major*, *Leishmania mexicana*, *Trypanosoma brucei* and *Toxoplasma gondii*.

In one study, the uptake of [^{3}H]adenine, [^{3}H]hypoxanthine, and [^{3}H]allopurinol, an antileishmanial hypoxanthine analogue, by *Leishmania major* promastigotes was investigated. The results showed that these compounds were all taken up by a single high-affinity transporter, LmajNBT1, with K_m values of 4.6 ± 0.9, 0.71 ± 0.07, and 54 ± 3 μM and V_{max} values of 3.2 ± 0.3, 2.8 ± 0.6, and 0.24 ± 0.06 pmol (10^7 cells)$^{-1}$ s^{-1}, respectively. [^{3}H]adenine transport was fully inhibited by the natural purines guanine and xanthine, with K_i values of 2.8 ± 0.7 and 23 ± 8 μM, respectively. Using purine analogues, an extensive inhibitor profile for LmajNBT1 was obtained, which allowed the construction of a quantitative model for the interactions between the transporter binding site and the permeant. The model predicts that hypoxanthine was bound through hydrogen bonds to N(1)H, N3, N7, and N(9)H of the purine ring, with a total Gibbs free energy of -39.5 kJ/mol. The interactions with adenine were similar, except for a weak hydrogen bond to N1 (unprotonated in adenine). The predicted model of substrate binding for LmajNBT1 was almost identical to that for the *Trypanosoma*

brucei H2 (TbH2) transporter. It is proposed that the architecture of their respective binding sites is very similar and that LmajNBT1 can be named a functional homolog of TbH2.

This thesis also reports the first identification and characterization of a purine nucleobase transporter in *Leishmania* amastigotes. Uptake of [^{3}H]hypoxanthine by *Leishmania mexicana* amastigotes was mediated by a single high-affinity transporter, LmexNBT1, with K_m and V_{max} values of 1.6 ± 0.4 μM and 0.092 ± 0.057 pmol (10^7 cells)$^{-1}$ s^{-1}, respectively. with high affinity for adenine, guanine, and xanthine, with K_i values of 4.2 ± 0.8, 1.7 ± 0.1, and 13 ± 2 μM, respectively, but low affinity for nucleosides and pyrimidine nucleobases. Allopurinol was apparently taken up by the same transporter (K_m of 33.6 ± 6.0 μM). All evidence was compatible with a model of a single purine nucleobase transporter being expressed in amastigotes. Using various purine nucleobase analogues, a model for the interactions between hypoxanthine and the transporter's permeant binding site was constructed and compared with previously obtained models for substrate recognition by nucleobase transporters, and found to be very similar to the models of the LmajNBT1 and TbH2 transporters, but markedly different from the human FNT1 transporter.

The work with procyclic forms of *Trypanosoma brucei* has led to the identification and characterization of a novel nucleobase transporter, designated H4, with K_m and V_{max} values of 0.55 ± 0.07 μM and 0.27 ± 0.08 pmol (10^7 cells)$^{-1}$ s^{-1}, respectively. The kinetic profile obtained from the characterization of H4 was identical to the one observed in a parallel study in our laboratory from the yeast-expressed nucleobase transporter TbNBT1. Moreover, introduction of tetracycline-inducible RNAi constructs in procyclic trypanosomes reduced H4-activity by ~90%. These findings confirmed that the *TbNBT1* gene encodes the first high affinity nucleobase transporter from protozoa to be identified at the molecular level and that H4 is likely to be the gene product of *TbNBT1*.

In the next chapter, we describe in great detail the functional characterisation of Adenosine Transporter B (AT-B) [or Nucleoside Transporter 10 (NT10)] and Adenosine Transporter D (AT-D) or [Nucleoside Transporter 9 (NT9)] expressed in yeast, and their expression in different developmental stages in the life cycle of *T. brucei*. Our observations confirm that NT10/AT-B is a P1-type transporter expressed specifically in short-stumpy bloodstream forms. The uptake of [^{3}H]adenosine and [^{3}H]inosine in *Saccharomyces cerevisiae* strain MG887-1 expressing AT-B, was rapid and saturable, exhibiting an apparent K_m values of 0.44 ± 0.02 and 0.52 ± 0.05 μM and V_{max} values of 0.60 ± 0.05 and 0.25 ± 0.05 pmol(10^7 cells)$^{-1}$s^{-1}, respectively. We also report a full characterisation of NT9/AT-D, which is also shown to be a P1-type transporter: it is predominantly a purine nucleoside transporter with at best a secondary capacity to transport nucleobases or pyrimidines. But NT9/AT-D displayed much higher affinity for adenosine than any other nucleoside transporter yet reported, with K_m values of 58 ± 12 nM and 2.7 ± 0.70 μM and V_{max} values of 0.011 ± 0.004 and 0.12 ± 0.039 pmol(10^7 cells)$^{-1}$s^{-1}, for adenosine and inosine, respectively. This high affinity of NT9/AT-D appeared to be a result of the transporter binding adenosine through interactions with both the P1 and P2 substrate recognition motifs.

Finally, purine transport in *T. gondii* has been investigated and novel nucleoside and nucleobase transporters were identified. The hypoxanthine carrier TgNBT1 (K_m of 0.91 ± 0.19 μM and V_{max} of 0.0045 ± 0.0014 pmol(10^7 cells)$^{-1}$s^{-1}) is inhibited by guanine (K_i = 6.9 μM) and xanthine (K_i = 134 μM). It is the first high affinity nucleobase transporter to be identified in an apicomplexan parasite. We have also identified a novel nucleoside transporter, TgAT2, which is high affinity and inhibited by adenosine (K_m of 0.49 ± 0.12 μM and V_{max} of 0.17 ± 0.04 pmol(10^7 cells)$^{-1}$s^{-1}), inosine (K_m of 0.77 ± 0.20 μM and V_{max} of 0.0048 ± 0.002 pmol(10^7 cells)$^{-1}$s^{-1}), guanosine (K_i = 1.5 ± 0.3 μM), uridine (K_i = 1.5 ± 0.4 μM) and thymidine (K_i = 1.3 ± 0.5 μM) as well as cytidine (K_i = 32 ± 10 μM). TgAT2 also recognises several nucleoside analogues with therapeutic potential. We have investigated the basis for the broad specificity of TgAT2 and found that hydrogen

bonds are formed with the 3' and 5' hydroxyl groups and that the base groups are bound through H-bonds with either N3 of the purine ring or N(3)H of the pyrimidine ring, and most probably π–stacking as well. The identification of these high affinity purine nucleobase and nucleoside transporters reconciles for the first time the low abundance of free nucleosides and nucleobases in the intracellular environment of the parasite with the efficient purine salvage carried out by *T. gondii*.

In summary, a survey of purine transport in many of the most important protozoan pathogens has been undertaken, identifying numerous new transport activities and characterising them in detail. At the same time, several putative purine transporters from *T. b. brucei* were cloned and expressed in heterologous systems. This enabled us to match these genes to specific transporter activities observed in live parasites and study their expression patterns in the life cycle.

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List of Abbreviations

AAT	animal african trypanosomiasis
AB	assay buffer
Ado	adenosine
AK	adenosine kinase
AIDS	acquired immune deficiency syndrome
AraA	adenine arabinoside
AT-A	adenosine transporter like-A
AT-A F	adenosine transporter like-A forward
AT-A R1	adenosine transporter like-A reverse
AT-B	adenosine transporter like-B
AT-D	adenosine transporter like-D
ATP	adenosine triphosphate
BF or BSF	bloodstream form
bp	basepair
CAFES	college of agricultural, food, and environmental sciences
CBSS	Carter's balanced salt solution
CCCP	carbonyl cyanide chlorophenylhydrazone
CDC	centers for disease control and prevention
cDNA	complementary deoxyribonucleic acid
Ci	curie
cib-type	concentrative insensitive to NBMPR (broad range of permeants)
cif-type	concentrative insensitive to NBMPR (formycin B as permeant)
cit-type	concentrative insensitive to NBMPR (thymidine as permeant)
CL	cutaneous leishmaniasis

CNS	central nervous system
CNT	concentrative nucleoside transporter
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DCL	diffuse cutaneous leishmaniasis
DEPC	diethyl pyrocarbonate
DFMO	difluoromethylornithine
dGTP	deoxyguanosine triphosphate
DMSO	dimethylsulphoxide
DNDi	drugs for neglected diseases initiative
dTTP	deoxythymidine triphosphate
E	epimastigotes
EATRO	east african trypanosmiasis research organisation
EDTA	ethylenediamine tetra acetic acid
ei	equilibrative insensitive
ENT	equilibrative nucleoside transporter
es	equilibrative sensitive
FCS	fetal bovine serum
h	hour
HAPT1	high affinity pentamidine transporter 1
HAT	human african trypanosomiasis
hCNT	human concentrative nucleoside transporter
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HGPRTase	hypoxanthine guanine phosphoribosyl transferase
HI-FCS	heat-inactivated fetal calf serum

HIV	human immunodeficiency virus
HXGPRT	hypoxanthine xanthine guanine phosphoribosyltransferase
IC_{50}	50% inhibitory concentration
Ino	inosine
IP	intraperitoneally
kb	kilobase
K_i	Inhibition constant
kJ	kilojoule
K_m	Michaelis-Menten constant
KO	knockout
LAPT1	low affinity pentamidine transporter 1
LdNT1	*leishmania donovani* nucleoside transporter 1
LmajNBT1	*leishmania major* nucleobase transporter 1
LmexNBT1	*leishmania mexicana* nucleobase transporter 1
MCL	mucocutaneous leishmaniasis
mCNT	mouse concentrative nucleoside transporter
mRNA	messenger RNA
mol	mole
MOPS	3-(N-Morpholino)propanesulfonic acid
MT	metacyclic trypomastigotes
mV	millivolt
n	number
NADH	nicotinamide adenine dinucleotide dehydrogenase
NBMPR	nitrobenzylthioinosine
ND	not determined

NE	no effect
nM	nanomolar
n.s	not significant
OD	optical density
ORF	open reading frame
RES	reticuloendothelial system
PC	procyclic
PCR	polymerase chain reaction
PF	procyclic form
Pfu	*Pyrococcus furiosus*
pmol	picomol
PSG	phosphate buffered saline plus glucose
PT	procyclic trypomastigotes
PV	parasitophorous vacuole
rCNT	rat concentrative nucleoside transporter
rpm	Revolutions per minutes
RT – PCR	reverse transcriptase- polymerase chain reaction
s	second
Sc	*saccharomyces cerevisiae*
SC-URA	synthetic complete minimal medium minus uracil
SDM	schneider's drosophila medium
SDS	sodium dodecylsulphate
SS	short stumpy
MBSU	molecular biology support unit
Taq	*Thermos aquaticus*

TbAT1	*trypanosome brucei* adenosine transporter 1
TBE	tris-borate-EDTA
TbH2	*trypanosome brucei* hypoxanthine transporter 2
TbNBT1	*trypanosome brucei* nucleobase transporter 1
TbNT	*trypanosome brucei* nucleoside transporter
TFGA	tasmanian farmers & graziers association
TgAT	*toxoplasma gondii* adenosine transporter
TgNBT	*toxoplasma gondii* nucleobase transporter
TgNT	*toxoplasma gondii* nucleoside transporter
TREU	trypanosome research Edinburgh university
U	unit
USDA	united states department of agriculture
UV	ultraviolet
V	volts
VL	visceral leishmaniasis
Vmax	maximum velocity
VSG	variant surface glycoprotein
WHO	world health organization
WNV	west nile viruses
w/o	without
WT	wild type
µl	microlitre
µg	microgram
µM	micromolar

Chapter One

GENERAL INTRODUCTION

1.1 Leishmaniasis

1.1.1 Disease

Leishmaniasis is a disease caused by the parasites of the genus *Leishmania*. The *Leishmania* protozoan was first described in 1903 by Leishman and Donovan, working separately (Herwaldt, 1999a). Since then, this organism has been found to be a complex grouping of species, at least 20 of which cause infections in humans. The disease is the cause of great suffering and death. There are 12 million people throughout the world that suffer from leishmaniasis, and the leishmaniases and the suffering they cause threaten 350 million men, women and children in 88 countries around the world, 72 of which are developing countries (Figure 1.1). The leishmaniases are related to environmental changes such as deforestation, building of dams, new irrigation schemes, urbanization and migration of non-immune people to endemic areas. It seriously hampers productivity and vitally needed socioeconomic progress and epidemics have significantly delayed the implementation of numerous development programmes. This is particularly true in Saudi Arabia, Morocco, the Amazon basin and the tropical regions of the Andean countries. Infection by *Leishmania* gives rise to a variety of clinical manifestations classically labelled as cutaneous, diffuse cutaneous, mucocutaneous and visceral, depending on the species, geographic region and host immune response (Pearson and Sousa, 1996; Pearson, *et al.*, 2001). The World Health Organization has reported that 90% of all visceral leishmaniasis occur in Bangladesh, Brazil, India and the Sudan, 90% of mucocutaneous leishmaniasis occur in Bolivia, Brazil and Peru, and 90% of cutaneous leishmaniasis cases occur in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria (World Health Organization 2002).

Visceral Leishmaniasis (VL) is known as Kala-azar and is the most serious form of the disease and can be fatal if untreated. The etiological agents belong to the *Leishmania donovani* complex, *L.d donovani, L.d infantum* and *L.d archibaldi* in the old world and

L.d chagasi in the new world. The old world species are transmitted by the sandfly vector (*Phlebotomus sp*). Humans, wild animals and domestic animals are known to act as reservoir hosts. *Lutzomyia logipalpis* is the only sandfly vector that has been implicated in the transmission of the new world species of visceral leishmaniasis and wild and domesticated dogs are known to serve as reservoir hosts (WHO expert committee report, 1991; Shiddo, *et al.,* 1995; Arias, *et al.,* 1996).

FIGURE 1.1 Geographical distribution of Leishmaniasis. Map of the world showing the endemic areas in 88 countries on 4 continents. Image was obtained from: WHO, TDR Disease website. http:www.who.int/tdr/diseases/leish/diseaseinfo.htm

The annual incidence of visceral leishmaniasis is estimated to be in the region of 500,000, 90% of which occurs in India, Nepal, Bangladesh, Sudan, and Brazil (Bora, 1999; Guerin, *et al.,* 2002; Desjeux, 2004; Gramiccia and Gradoni, 2005). In Sudan, an epidemic killed 100,000 people between 1984 and 1996 (Seaman, *et al.,* 1996). In Brazil, the disease is a common cause of admission to hospital (Costa, *et al.,* 1990), and visceral leishmaniasis cases were 1840 and 6000 in 1998 and 2002, respectively (Gramiccia and Gradoni, 2005). In southern Europe co-infection with HIV, which has led to difficulties with diagnosis and treatment, is changing the epidemiological pattern of the disease

and may be introducing a new, human, reservoir of infection (Alvar, *et al.*, 1997; Capelli, *et al.*, 2004). In the Mediterranean basin 1.5-9% of AIDS patients develop visceral leishmaniasis and 25-70% of the adult visceral leishmaniasis cases are related to HIV infection. Many of the cases are in children. In India, 40% of patients are aged <13 years (Bora, 1999). In Sudan, 65% are aged <15 years (World Health Organization, 1998), and in Brazil, 60% of patients are aged <5 years (Desjeux, World Health Organization, 1991). The new world and the old world forms of the disease display similar symptoms and are often complicated by secondary infections (WHO www website, 1997). It is a generalized infection of the reticuloendothelial system (RES) involving the spleen, liver, bone marrow and lymph nodes. The disease is characterized by fever, hepatomegaly, enlarged lymph nodes and tonsils. Patients often exhibit a wasting syndrome despite good appetite as the disease progresses. Other possible manifestations include oedema, anaemia, leucopaenia, monocytoses, lymphocytoses, and thrombocytopaenia (Figure 1.2). Inadequate treatment can lead to cutaneous disease known as post kala azar dermal leishmaniasis characterized by nodular lesions similar to diffuse cutaneous leishmaniasis. In contrast to DCL, this post kala azar is easily cured with treatment.

FIGURE 1.2 Child with Visceral Leishmaniasis in Bangladesh. Image was obtained from Guerin, *et al.* (2002).

Cutaneous leishmaniasis (CL) is the most common manifestation of the disease. These are generally benign self-healing lesions that are painless and non-pruritic and take weeks to months to heal. Quite often the lesions are ulcerated with a raised border and without pus. However, the lesions can become secondarily infected with bacteria. These simple cutaneous lesions can also be of a popular or nodular nature. The lesion represents a localized infection at the site of the sandfly bite. Satellite lesions in the vicinity of the original lesion are sometimes observed. In the Gulf War (1990 to 1991), approximately 19 cases of cutaneous leishmaniasis were diagnosed in the U.S troops (William, *et al.,* 2004), and about 150 cases of leishmaniasis have reportedly been diagnosed in U.S. soldiers serving in Iraq in 2003 (McNeil, 2003), Preliminary data on 22 cases of cutaneous leishmaniasis contracted by American troops in Afghanistan, Kuwait, and Iraq and treated at Walter Reed Army Medical Center between August 2002 and September 2003 were recently released (Centers for Disease Control and Prevention, CDC, 2003). The majority of these persons were infected with *Leishmania major* in urban areas of Iraq after a median period of deployment of 60 days.

More than 90% of the world's cases of cutaneous leishmaniasis are in Afghanistan, Algeria, Brazil, Iran, Iraq, Peru, Saudi Arabia, and Syria (Murray, *et al.*, 2005). However, approximately 75% of the cases that are evaluated in the United States were acquired in Latin America, where leishmaniasis occurs from northern Mexico (rarely in rural southern Texas) to northern Argentina. In Brazil, cutaneous leishmaniasis cases were 21,800 in 1998, while there were 40,000 in 2002 (Gramiccia and Gradoni, 2005). Leishmaniasis is not found in Australia or the South Pacific. Kabul is the largest centre of cutaneous leishmaniasis in the world, with an estimated 67,500 cases. The figure accounts for a third of the 200,000 cases in all of Afghanistan. At the same time, the influx of large numbers of displaced people threatens to increase the disease's already epidemic levels. With little immunity to leishmaniasis, displaced people, or in this case, people returning from neighbouring Pakistan, are typically more susceptible to the disease. (World Health Organization, News, 2004). Cutaneous leishmaniasis occurs in

the New World and the Old World. Old World disease is primarily caused by *Leishmania tropica* in urban areas and *Leishmania major* in dry desert areas. The two subgenera of interest in Latin America are *Leishmania leishmania* (e.g., *Leishmania mexicana, Leishmania amazonensis, and Leishmania chagasi*) and *Leishmania viannia* (e.g., *Leishmania panamensis, Leishmania braziliensis, and Leishmania guyanensis*). The incubation period is two to eight weeks, although longer periods have been noted. The disease begins as an erythematous papule at the site of the sandfly bite on exposed parts of the body. The papule increases in size and becomes a nodule. It eventually ulcerates and crusts over. The border is usually raised and distinct. There may be multiple lesions, especially when the patient has encountered a nest of sandflies. The ulcer is typically large but painless unless there is secondary bacterial or fungal infection (Markle and Makhoul, 2004).

Mucocutaneous leishmaniasis (MCL) is also called mucosal form and usually occurs after an initial cutaneous infection. Ninety percent of cases of mucosal leishmaniasis are found in the tropics of South America Brazil, Bolivia, and Peru, and they usually begin in the nose or palate (WHO report, 2003). This manifestation is primarily due to members of the *L. braziliensis* complex (Gramiccia and Gradoni, 2005). Mucocutaneous disease begins as simple skin lesions that metastasize via the blood stream or lymphatics, particularly to the mucosae of the nose and mouth, other symptoms include fever, weight loss, and anemia. The expression of this form of the disease can occur several years after the primary lesion. This disease will generally continue to progress and can lead to severe pathology and even deformity if not treated. Although some patients may simultaneously exhibit skin and mucosal lesions, it has been observed that in the majority of cases (59%) from Amazonian Brazil the mucosal disease has resulted from an old, prolonged, and untreated (self-healing) cutaneous infection with *L. (V.) braziliensis* (Silveira, *et al.*, 1999). This parasite is recognized as the most important etiologic agent of mucocutaneous leishmaniasis in the New World (Lainson, 1983; Grimaldi, *et al.*, 1987; Lainson and Shaw, 1998).

Diffuse cutaneous leishmaniasis (DCL) and leishmaniasis recidivans are two rare manifestations of cutaneous leishmaniasis. This disease is characterized by disseminated nodular lesions that resemble lepromatous leprosy. These lesions tend to be scaly and not ulcerated. Recivida is a chronic recurrence of nodular lesions or a rash characterized by hypersensitivity. Neither diffuse cutaneous leishmaniasis nor recidiva is easily cured. This type of disease occurs most often in Ethiopia, Brazil, Dominican Republic, and Venezuela. The lesions of diffuse cutaneous leishmaniasis are very similar to those of localized cutaneous leishmaniasis, except they are spread all over the body. The patient's immune system apparently fails to battle the protozoa, which are free to spread throughout the body. In the New World, diffuse cutaneous leishmaniasis has been associated with *Leishmania pifanoi* (Schnur, *et al.*, 1983), *L. amazonensis*, *L. mexicana* (Simpson, *et al.*, 1968; Grimaldi, *et al.*, 1989; Gramiccia and Gradoni, 2005), and *L. venezuelensis* (Bonfante-Garrido, *et al.*, 1992). In the Old World it has been associated with *L. aethiopica* (Grevelink and Lerner, 1996), *L. major* (Morsy, *et al.*, 1997), and *L. tropica* (Takahashi Sato, 1981).

1.1.2 Organisms and life cycle

Leishmaniasis is a disease complex caused by species of haemoflagellate protozoan parasites belonging to the genus *Leishmania*, which belong to the order Kinetoplastida, family Trypanosomatidae. *Leishmania* spp are divided into two subgenera: the *Viannia* subgenus includes *L. (Viannia) braziliensis* and related species that develop in the hindgut before migrating to the midgut and foregut. The other subgenus such as *L. (leishmania) donovani*, occupy only the midgut and foregut (Lainson and Shaw, 1987). Morphologically, *leishmania* is divided in to two forms. Amastigotes are spherical in shape, only about 2-3 μm in diameter, and they reside and multiply within phagolysosomes in mammalian mononuclear phagocytes. There is a prominent nucleus and kinetoplast, and the cytoplasm is vacuolated and contains lysosomes. The outer

membrane has a polysaccharide component but there is no surface coat (Lindsay, *et al*, 2002). Promastigotes are similar in structure, apart from the prominent flagella.

Leishmaniasis is transmitted through the bite of female phlebotomine sandflies in Mediterranean Europe, North Africa, East Africa, India and China (Figure 1.3). In South America, transmission is by the genus *Lutzomyia* (Rogers, *et al.*, 2004; Murray, *et al.*, 2005). Sandflies feed on plant juices but for the most part the females need a blood-meal in order to develop eggs. Autogeny occurs in a few species. Blood is taken from humans and animals such as dogs, farm livestock, wild rodents, snakes, lizards and birds. Each sandfly species has specific preferences for its source of blood, but the availability of hosts is an important factor. The saliva of sandflies can enhance the virulence of inoculated *Leishmania* parasites (Titus and Ribeiro, 1988; Theodos, *et al.*, 1991). Sandfly species are only important as vectors of leishmaniasis if they feed regularly on humans.

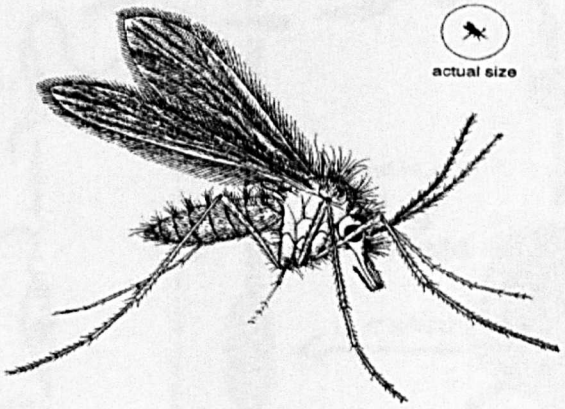


FIGURE 1.3 Phlebotomine sandflies. 1.3 - 3.5 mm in length, hairy appearance, conspicuous black eyes, long, stilt-like leg. Obtained from: WHO documents 1997: www.who.int/entity/water_sanitation_health/resources/vector007to28.pdf

The sandfly vector becomes infected when feeding on the blood of an infected individual or an animal host. The *leishmania* parasites live in the macrophages as

round, non-motile amastigotes. The macrophages are ingested by the fly during the blood- meal and the amastigotes are relesed into the vector midgut. These parasites then differentiate into the motile, elongated (10-20 micrometers), flagellate metacyclic promastigote form which takes 1-2 weeks. The mature metacyclic promastigotes then migrate to the alimentary tract of the fly, where they live extracellularly and multiply by binary fission (Schmidt and Roberts, 1989). Four to five days after feeding the promastigotes move forward to the salivary glands of the insect. When the sandlfy next feeds on a mammalian host, promastigote forms of the leishmanial parasite enter the mammalian host via the proboscis (Figure 1.4). Once in the host the promastigotes are taken up by the macrophages where they rapidly revert to the amastigote form within the phagolysosome by losing its flagellum and becoming more spherical (Bates and Rogers, 2004; Rogers, *et al.*, 2004).

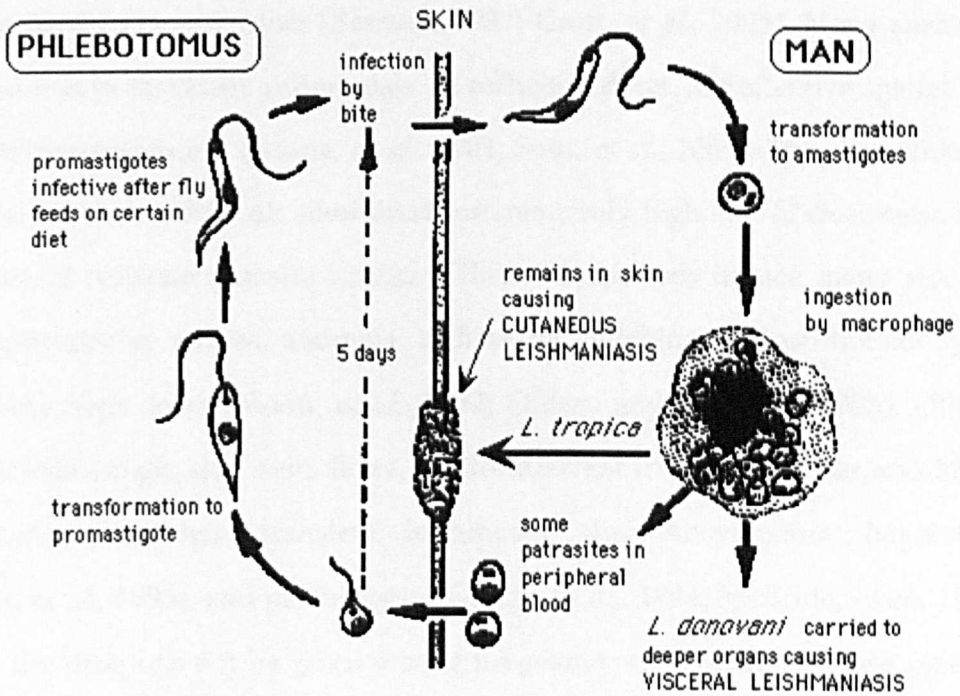


FIGURE 1.4 Diagrammatic representation of the *Leishmania* life cycle. Obtained from: Protozoa as Human Parasites website: http://www-micro.msb.le.ac.uk/224/Parasitology.html.

The *Leishmania* are able to resist the microbiocidal action of the acid hydrolases releaseed into the lysozymes and so survive and multiply inside the macrophages, eventually leading to the lysis of the macrophages. The released amastigotes are taken up by additional macrophages and so the cycle continues. Ultimately all the organs containing macrophages and phagocytes are infected, especially the spleen, liver and bone marrow.

1.1.3 Therapy

The treatment of leishmaniasis depends on the infecting *leishmania* sp, and the clinical syndrome (Pearson and Sousa, 1996; Berman, 1997). Pentavalent antimonial compounds, either sodium stibogluconate (Pentostam) or meglumine antimoniate (Glucantime), have remained the first choice of drug in the treatment of all forms of leishmaniasis for many decades (Berman, 1997; Croft, *et al.*, 2005). Many studies have confirmed that pentavalent antimonials, in sufficient doses, are effective against several types of leishmaniasis e.g. (Arana, *et al.*, 2001; Soto, *et al.*, 2005). They are widely used despite their toxicity, difficult administration, relatively high cost of treatment, and the appearance of resistant parasite strains. These compounds induce many side effects including headache, nausea, anorexia, arthralgias, phlebitis, and cardiotoxicity when used in very high doses (Navin, *et al.*, 1992; Olliaro and Bryceson, 1993). Other side effects include cough, skin rash, fever, gastrointestinal irritation (Wyler and Marsden, 1984), malaise, myalgia, transient leucopenia, thrombocytopenia, hepatotoxicity (Hepburn, *et al.*, 1993), and pancreatitis, (Gasser, *et al.*, 1994; McBride, *et al.*, 1995). In addition, the drug can not be given during pregnancy and requires a close supervision when administered to patients with cardiac problem. The limitations on the use of these drugs have stimulated a widespread interest in evaluating alternative drugs.

The polyene antibiotic amphotericin B is currently a second-line treatment for visceral leishmaniasis (VL) and mucocutaneous leishmaniasis. The use of this drug is fraught with well-known adverse effects including fever, malaise, nausea, vomiting, nephrotoxicity, and phlebitis. In order to reduce this dose-limiting toxicity, different commercial lipid-based formulations have been developed and used in the clinical management of VL, AmBisome (Davidson, *et al.*, 1991; Berman, *et al.*, 1998), Abelcet (Sundar and Murray, 1996; Sundar, *et al.*, 1998a), and Amphotec (Dietze, *et al.*, 1993). These have also been used as alternative treatments for mucocutaneous leishmaniasis (Zijlstra, *et al.*, 2003). In some developed countries, AmBisome is now indicated as the first-line therapy against VL (Sundar, *et al.*, 2003; Croft, *et al.*, 2005). However, the high prices of these formulations restrict their use in the regions most affected by these tropical diseases (Lockwood, 1998; Guerin, *et al.*, 2002). New lipid formulations of amphotericin B exhibit much less toxicity, but are extraordinarily expensive by developing world standards. Lipid-amphotericin B formulations with lower toxicity than the parent drug that were developed for the treatment of systemic mycoses have proved to be an effective treatment for VL, especially AmBisome. This drug is named a "miracle drug" because it is simple to use (maximum of 10 days), revives patients within hours of getting the first shot, and has virtually no side effects. The drawback is that there is only one producer and it is astronomically expensive – the best current price offer is US$1,500-2,400 per treatment, well beyond the reach of the average patient. *In vitro*, free amphotericin B was three to six times more active than the liposomal formulation AmBisome against both *Leishmania major* promastigotes in culture and amastigotes in murine macrophages. It has been suggested that liposomal amphotericin B may be useful in the treatment of cutaneous Leishmaniasis (Yardley and Croft, 1997). In dogs naturally infected with *L. infantum* were treated with liposomal amphotericin B (Oliva, *et al.*, 1995; Lamothe, 2001). Despite nephrotoxicity, amphotericin B is being increasingly used both as the drug of choice for human visceral leishmaniosis in patients with human immunodeficiency virus infection, and in cases where there is pentavalent antimonial resistance (Walker, *et al.*, 1998).

Pentamidine was tried for *leishmania* in an attempt to limit side-effects of systemic antimony. The drug was highly effective and acceptably tolerated as an alternative to antimony in the treatment of cutaneous Leishmaniasis in the New World (Ouellette, *et al.*, 2004), but was used in only one study in the Old World (Hellier, *et al.*, 2000). Pentamidine isethionate is associated with many adverse effects, such as headache, hypotension, nausea, vomiting, and hypoglycemia followed by diabetes mellitus and the development of drug resistance (Thakur, *et al.*, 1995; Jha, *et al.*, 1998; Sundar, *et al.*, 1998a, Sundar, 2001a).

Allopurinol is a drug in use for the treatment of gout. It is supposed to function as an alternative substrate for the enzyme Hypoxanthine Guanine Phosphoribosyl Transferase (HGPRTase), so allowing the formation of allopurinol riboside monophosphate, and its incorporation into RNA, which leads to the inhibition of protein synthesis in the parasite (Marr, *et al.*, 1982). It is also on trial for the treatment of Chagas disease and the drug is now widely used for the treatment of leishmaniasis in dogs. Allopurinol monotherapy has no effect on Colombian cutaneous disease primarily caused by *L. panamensis* and therefore is unlikely to be effective against cutaneous leishmaniasis in other endemic regions (Velez, *et al.*, 1997). Allopurinol, a purine analogue, has been used to treat leishmaniasis alone (Pfaller and Marr, 1974; Marsden, *et al.*, 1984), or combined with other drugs such as antimonial compounds (Chunge, *et al.*, 1985; Martinez and Marr, 1992; Momeni and Aminjavaheri, 1995; Martinez, *et al.*, 1997; Denerolle and Bourdoiseau, 1999; Momeni, *et al.*, 2002; Pasa, *et al.*, 2005). A novel combination uses ketoconazole and allopurinol, two drugs that have been individually suggested as second line treatments for visceral leishmaniasis (Hueso, *et al.*, 1999). Low cost, stability, safety, ease of administration (oral), and lack of toxicity make allopurinol a particularly appealing drug for tropical and subtropical diseases.

Miltefosine is the most recent treatment to come to market, which has the advantage of being orally active in contrast to almost all the other drugs (Sundar, *et al.*, 2002; Croft,

et al., 2005). This anti-cancer drug was discovered to be effective against kala-azar in mid-1990s, but has only been registered for use in India. However, extreme caution must be observed when administering this drug to women of child-bearing potential, and it is too expensive for most of the developing world. Orally administered miltefosine appears to be an effective treatment for Indian visceral leishmaniasis (Jha, *et al.*, 1999) and a promising oral drug for cutaneous leishmaniasis (Arana, *et al.*, 2001). Despite the recent success with miltefosine as an antileishmanial drug (Herwaldt, 1999b; Soto, *et al.*, 2001), its use is limited because of the risk of the rapid development of resistance if it is used alone (Bryceson, 2001).

Ketaconazole is a drug for fungal infections, but also inhibits sterol synthesis in *Leishmania* and interferes with the growth and division intracellular amastigotes. It is on trial for the use in dogs and in humans. Ketaconazole and itraconazole, have also been tested for cutaneous leishmaniasis, but results obtained were not completely satisfactory (Arana, *et al.*, 2001; Storer and Wayte, 2005). Although the ketoconazole tablet has been reported to be effective in the treatment of some cases of cutaneous leishmaniasis, the low response rate in patients receiving ketoconazole cream indicates that it cannot be used as the single agent in the treatment of cutaneous leishmaniasis patients (Momeni, *et al.*, 2003) or for visceral leishmaniasis when tested in *L. infantum* as an experimental model (Gangneux, *et al.*, 1999).

Paromomycin is a new potential drug for leishmaniasis, the effectiveness of this old antibiotic against kala-azar and cutaneous Leishmaniasis (Croft, *et al.*, 2005; Jha, *et al.*, 2005) was discovered accidentally in the 1960s. But the drug got stuck in the research pipeline, as it "would not make a lot of money". Moreover, its current formulation has not yet been registered for use anywhere in the world. However, WHO/TDR and Institute of One World Health (IOWH) are working to get the drug registered for use in India. In addition, the Drugs for Neglected Diseases Initiative (DNDi) Foundation is

launching clinical trials of the anti-leishmanial drug paromomycin for registration in Africa, (DNDi Press Release, Geneva 2004).

1.1.4 Drug resistance

The recent emergence of widespread clinical resistance in *leishmania* parasites is a major problem to their control. Drug resistance has great public health importance for anthroponotic *Leishmania donovani* and *Leishmania tropica.* Most other forms of leishmaniasis are zoonotic and so the spread of drug resistance is less likely (Croft and Coombs, 2003). The lack of response to pentavalent antimonials in visceral leishmaniasis has been a problem for many years and is increasing world wide, occurring in about 5-70% of the patients in some endemic areas in some developing nations (Ouellette and Papadopoulou, 1993; Guerin, *et al.,* 2002). In the state of Bihar in north-western India, up to 70% of *Leishmania* cases are resistent to antimonials (Sundar, 2001; Croft, *et al.,* 2006). The HIV epidemic is spreading from the towns into the country villages and is likely to make the situation worse and drug resistance is the priority. In Bangladesh, Brazil, resistance continues to grow (Seaman, *et al.,* 1996), and is spreading along the borders with Ethiopia (WHO Report, 2002). A relatively high proportion of patients with visceral leishmaniasis fail to respond to pentamidine in Kenya (Wijers, 1974), India (Thakur, *et al.,* 1993; Giri, 1994; Sundar, 2001), and China (Akhtar, and Humber, University of East London' website) due to increased resistance (Dietze, *et al.,* 1995; Thakur, *et al.,* 1995).

Resistance to paromomycin (aminosidine) has not yet been reported, but experience with its antibacterial usage suggests that the potential is there if it were used widely on its own (Bryceson, 2001). It has been reported that impairment in miltefosine (HePC) uptake is likely to be the main mechanism of resistance in *L. donovani* promastigotes (Perez-Victoria, *et al.,* 2003). In developed Mediterranean nations, drug-resistant

leishmaniasis continues to spread as the number of patients co-infected with HIV increases. Primary resistance to amphotericin B has not been reported and secondary resistance only in HIV co-infected patients. Isolates from HIV co-infected adult patients in France were increasingly resistant to amphotericin B following relapse after treatment (Di Giorgio, *et al.*, 1999). The mechanism of resistance to antimony in field strains is unknown. However, some studies of the drug resistance *in vitro* have suggested that there are many reasons, including reduced accumulation of the drug (Dey, *et al.*, 1994; Brochu, *et al.*, 2003). It has been reported that the resistance to paromomycin might be due to decreased drug uptake. A sensitive method to detect and to quantify intracellular paromomycin was developed showing that the main mechanism of resistance seems to be due to decreased drug uptake probably as a consequence of altered membrane composition (Maarouf, *et al.*, 1998).

1.2 African Trypanosomiasis

1.2.1 Disease

African trypanosomiasis or sleeping sickness is a disease caused by infection with protozoan parasites of the genus *Trypanosoma*, which enter the blood-stream via the bite of bloodfeeding tsetse flies (*Glossina spp.*). Trypanosomiasis remains as much a major public health hazard today as it did when studies first began more than 100 years ago (Hide, 1999). The disease affects both people (Human African Trypanosomiasis, HAT or sleeping sickness) and animals (Animal African Trypanosomiasis AAT, or nagana) and occurs in 37 sub-Saharan countries covering more than 9 million km^2, an area which corresponds approximately to one-third of the Africa's total land area, 22 of these countried are among the least developed countries in the world (Figure 1.5). The infection threatens an estimated 60 million men, women and children (Molyneux, 1997), and about 50 million head of cattle. However, only 3 to 4 million of these people are under surveillance and the 45.000 cases reported in 1999 do not reflect the reality of the situation, but simply show the absence of case detection. The estimated number of people thought to have the disease is between 300 000 and 500 000 (WHO report, 2001).

Human African trypanosomiasis (HAT) is a disease caused by two subspecies of *Trypanosoma brucei. Trypanosoma brucei gambiense,* which causes Gambian or chronic sleeping sickness, mainly in West and Central Africa. *Trypanosoma brucei rhodesiense* causes Rhodesian or acute sleeping sickness in East and South-central Africa (Balakrishnan and Zumla, 2001; Pepin and Meda, 2001). The parasites first develop in the blood, lymph and peripheral organs, which is known as stage one (Barrett, *et al.,* 2003), the main clinical signs of human trypanosomiasis are high fever, weakness, headache, joint pains and pruritus (itching). Gradually, the immune defence mechanisms and the patient's resistance are exhausted. As the parasite develops in the

lymph and blood of the patient, the initial symptoms become more pronounced and other manifestations such as anaemia, cardiovascular and endocrine disorders, and abortion, oedema and kidney disorders appear.

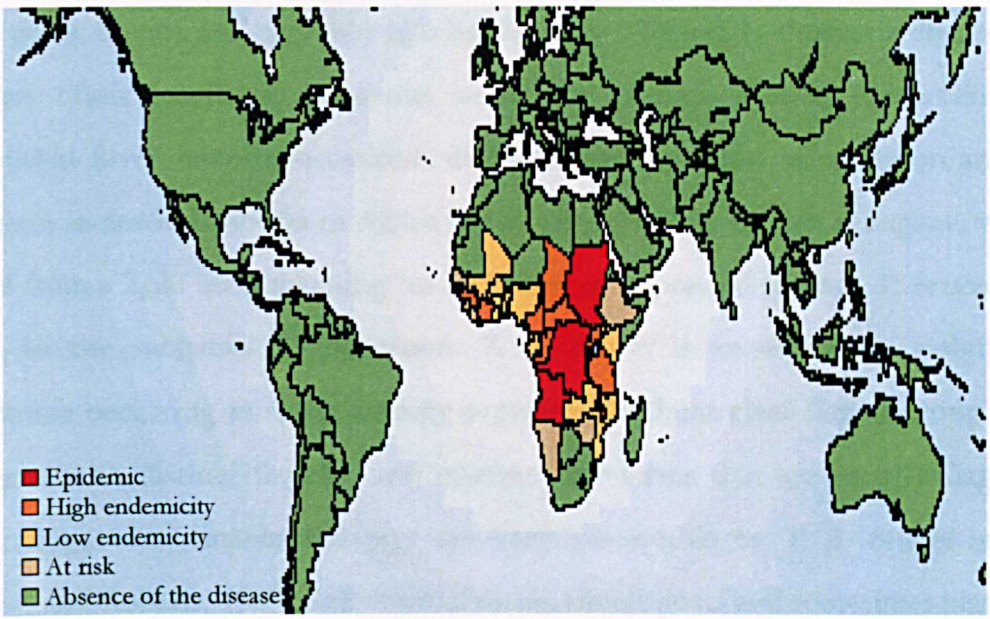


FIGURE 1.5 Map of the world showing the distribution of human African trypanosomiasis. Image was reproduced from: WHO, TDR disease. http://www.who.int/tdr/dw/tryp2004.htm.

In advanced stages of disease, the parasite invades the central nervous system (Chimelli and Scaravilli, 1997; Enanga, *et al.*, 2002). The patient's behaviour changes, they can no longer concentrate and become indifferent to their environment. Sudden and unpredictable mood changes become increasingly frequent, giving rise to lethargy with bouts of aggressiveness. Patients are overcome by such extreme torpor that eating, speaking, walking or even opening the eyes call for an unsurmountable effort. At night they suffer insomnia and during the day are exhausted by periods of sleep-like unconsciousness. Finally, patients fall into a deep coma and die, this occurs mainly in east and southern Africa.

African animal trypanosomiasis (AAT) is caused by genus *Trypanosoma congolense* resides in the subgenus *Nannomonas*, a group of small trypanosomes with medium-sized marginal kinetoplasts, no free flagella, and poorly developed undulating membranes. In east Africa, *T. congolense* is considered to be the single most important cause of AAT. This parasite is also a major cause of the disease in cattle in West Africa. Sheep, goats, horses, and pigs may also be seriously affected. In domestic dogs, chronic infection often results in subacute, acute, or chronic disease characterized by intermittent fever, anemia, occasional diarrhea, and rapid loss of condition and often terminates in death. In southern Africa the disease is widely known as nagana, which is derived from a Zulu term meaning "to be in low or depressed spirits". *T. brucei brucei* resides in the subgenus *Trypanozoon. T. b. brucei* is an extremely polymorphic typanosome occurring as short, stumpy organisms without clear flagella, long slender organisms with distinct flagella, and intermediate forms that are usually flagellated. Horses, dogs, cats, camels and pigs are very susceptible to *T. b. brucei* infection (Schmidt and Roberts, 1989). Infection of cattle, sheep, goats and sometimes pigs results in mild or chronic infection. This last observation, although widely accepted, has been called into question by Moulton and Sollod, who cite evidence that this organism is widespread in east and west Africa and that it can cause serious disease and high mortality in cattle, sheep, and goats (Moulton and Sollod, 1976). *T. vivax* is a member of the subgenus *Duttonella*, a group of trypanosomes with large terminal kinetoplasts, distinct free flagella, and inconspicuous undulating membranes. *T. vivax* is a large (18-26 μm long) monomorphic organism that is very active in wet-mount blood smears. Cattle, sheep, and goats are primarily affected (Roeder, *et al.*, 1984). Although this organism is considered to be less pathogenic for cattle than *T. congolense*, it is nevertheless the most important cause of AAT in West African cattle. This trypanosome readily persists in areas free of tsetse flies (for example, in Central and South America and in the Caribbean), where it is transmitted mechanically by biting flies or contaminated needles, syringes, and surgical instruments.

A few species of *Trypanosoma* are also found in the New World. From the standpoint of human health, the most important is *Trypanosoma cruzi* which causes American trypanosomiasis or Chagas disease. Chagas disease is found throughout much of central and northern South America, Central America, and Mexico. *T. cruzi* is found in a number of animals other than humans, including dogs, cats and rodents, but it is not known how often infections in these animals are transmitted to humans.

1.2.2 Organisms and life cycle

The parasite is an elongated cell with single nucleus which usually lies near the centre of the cell. Each cell bears a single flagellum which appears to arise from a small organelle of the kinetoplast. The kinetoplast is a specialised part of the mitochondria and contains DNA. The length and position of the trypanosome's flagellum is variable. In trypanosomes from the blood of a host the flagellum originates near the posterior end of the cell and passes forward over the cell surface, its sheath is expanded and forms a wavy flange called an undulating membrane (Figure 1.6).

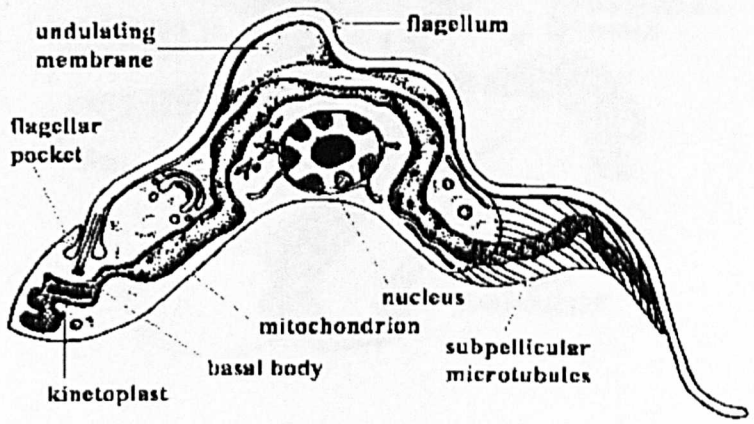


FIGURE 1.6 Diagram to show principal structures revealed by the electron microscope in the epimastigote stage of *Ttypanosoma,* Reproduced from Mark F. Wiser, Tulane University 1999. http://www.tulane.edu/~wiser/protozoology/notes/kinet.html#cycle

African trypanosomes are transmitted by several species of tsetse flies of genus *Glossina*. The tsetse fly becomes infected with bloodstream trypomastigotes when taking a blood meal on an infected mammalian host. The trypanosomes in the vertebrate's blood migrate into the vector's midgut, where the short stumpy forms complete the development of their mitochondrion and change their surface coat to differentiate into the long, slender procyclic trypomastigotes. As the procyclic stages have a fully developed mitochondrion and polysomes highly loaded with mRNA, they exhibit significantly higher levels of metabolic activity and protein synthesis than do the bloodsteam stages (Brecht and Parsons, 1998). They multiply by binary fission, leave the midgut, and transform into epimastigotes. The epimastigotes reach the fly's salivary glands and continue multiplication by binary fission. Eventually, some epimastigotes undergo a final transformation stage into non-dividing metacyclic trypomastigotes, which are short, stumpy, and highly motile. They lack free flagella, and have a terminally located kinetoplast. The cycle in the fly takes approximately 3 weeks (Figure 1.7).

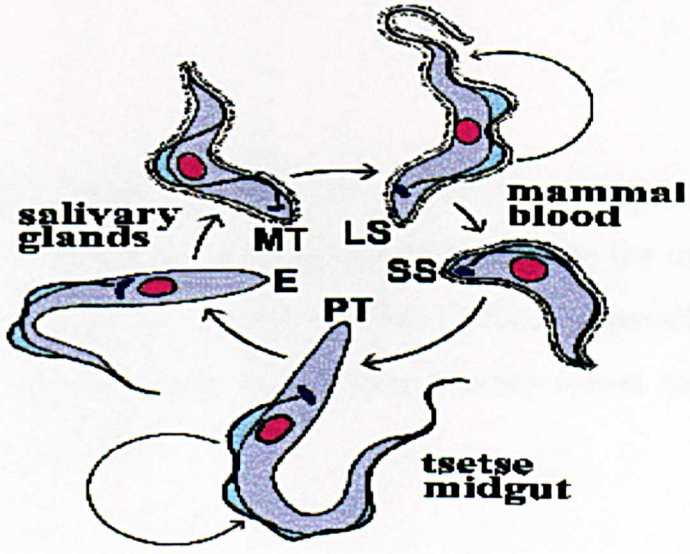


FIGURE 1.7 Diagram represents the life cycle of *Typanosoma*. SS: Short Stumpy bloodstream forms, PT: Procyclic Trypomastigotes, E: Epimastigotes, MT: Metacyclic Trypomastigotes, and LS: Long Slender bloodstream forms. Reproduced from Mark, F. Wiser, Tulane University 1999: http://www.tulane.edu/~wiser /protozoology/notes/kinet.html#cycle.

During a blood meal on the mammalian host, an infected tsetse fly (genus *Glossina*) injects metacyclic trypomastigotes into skin tissue. The parasites enter the lymphatic system and develop into the long slender bloodstream forms. Inside the host, they transform into bloodstream trypomastigotes, are carried to other sites throughout the body, reach other blood fluids (e.g., lymph, cerebro-spinal fluid), and continue the replication by binary fission until a threshold population is reached, whereupon a switch occurs, resulting in a subpopulation of long slender bloodstream forms transforming first into intermediate forms and then into short stumpy bloodstream forms. This transition involves cell cycle arrest and a decrease in protein synthesis, as evidenced by a reduction in ribosome loading (Brecht and Parsons, 1998). The SS forms are morphologically and functionally very different from the long slender bloodstream forms as they do not divide and have no free flagellum. It is likely that the short stumpy bloodstream form is the infective stage for the tsetse, and the switch from a predominance form of long slender bloodstream forms to short stumpy bloodstream forms is therefore essential for the cycle to continue (Seed, 1998).

1.2.3 Therapy

The use of drugs for the prevention and treatment of trypanosomiasis has been important for many decades. The drug regimen depends on the infecting species and the stage of infection. Treatment has always been difficult, especially when the disease has reached an advanced stage with central nervous system involvement, as few effective drugs are available.

Diamidines, including pentamidine and berenil (diminazene aceturate), have been first-line drugs for the treatment of early stage African trypanosomiasis for decades (Pepin and Milord, 1994; Croft, *et al.*, 2005). Pentamidine isethionate and suramin, under an investigational New Drug Protocol from the Centers for Disease Control (CDC) drug service, are used before the Central Nervous System (CNS) involvement,

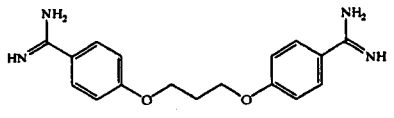
and are the drugs of choice to treat the hemolymphatic stage of West and East African Trypanosomiasis (Croft, *et al.*, 2005). However, Pentamidine is not effective against late-stage disease and Suramin has to be administered intravenously and can have adverse side-effects. Berenil is a veterinary drug that has also been used occasionally without licence (Barrett, *et al.*, 2003). Melarsoprol, an arsenical drug developed over 50 years ago, is the drug of choice for late-stage disease with central nervous system involvement, but often induces serious, sometimes fatal side effects.

A newer drug, eflornithine, originally developed as an anticancer agent, has shown to be effective against the *T. b. gambiense* form. This polyamine synthesis inhibitor difluoromethylornithine (DFMO) has emerged as an alternative therapy when the outlook has hitherto been grim. This drug is used after the failure of the usual first-line treatment Melarsoprol (Milord, *et al.*, 1992; Khonde, *et al.*, 1997; Na-Bangchang, *et al.*, 2004; Croft, *et al.*, 2005), and because it is very expensive, a complete treatment costs US$ 250, making its use as a first choice drug almost impossible in Africa (Aksoy, 2003).

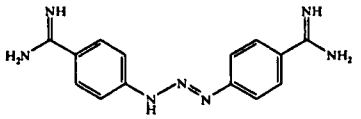
Some of the older chemoprophylactic drugs such as the quinapyramine derivatives Antrycide and Antrycide prosalt are still used and give effective protection against *T. b. brucei* infection in horses, camels, and cattle for a short time. Quinapyramine derivatives are the most commonly used trypanocidal drug in China. Drug resistance in *T. evansi* isolates has been reported from Africa (Boid, *et al.*, 1989; El Rayah, *et al.*, 1999) and the far east of Asia (Dieleman, 1986).

The drug pyrithidium bromide (Prothidium and AD2801) is useful in the prophylaxis of *T. vivax* and *T. congolense* infections in cattle, sheep, and goats and can give protection for up to 6 months. The most widely used of the chemoprophylactic drugs is isometamidium chloride which has been in use for over 20 years and sold under the trade names Samorin and Trypamidium, is excellent for the prophylaxis of all three African animal trypanosomes, and gives protection for 3-6 months. Homidium bromide

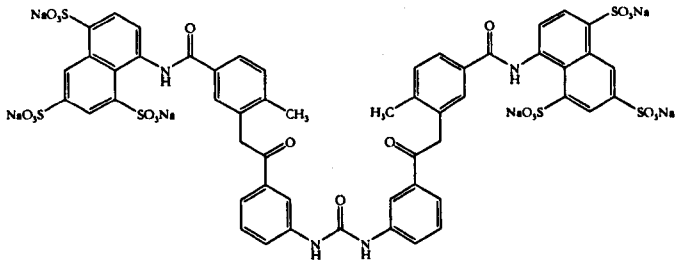
has also been found to be an effective chemophrophylactic drug in Kenya, and both homidium and isometamidium chloride are drugs in use against trypanosomiasis in cattle in the African countries (Stevenson, *et al.*, 1995).



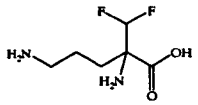
Pentamidine isethionate (Diamidine)



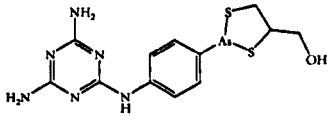
Berenil (Diminazene aceturate)



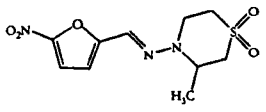
Suramine (Moranyl®)



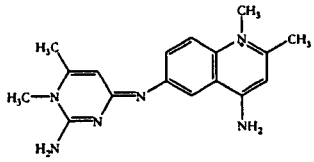
Difluoromethylornithine, DFMO (Eflornithine®)



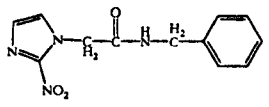
Melarsoprol (Arsobal®)



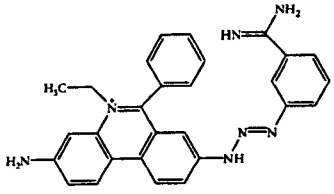
Nifurtimox (Lampit®)



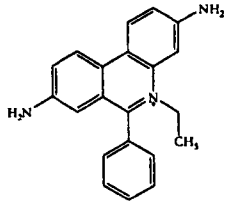
Antrycide



Benznidazole (Radinil®)



Isometamidium chloride (Samorin)



Homidium bromide

FIGURE 1.8 Structural formulas of drugs used against Human African Trypanosomiasis (HAT) and Animal African Trypanosomiasis (AAT).

In addition, control relies mainly on systematic surveillance of at-risk populations, coupled with treatment of infected people. Reduction of tsetse fly numbers plays a significant role, especially against the rhodesiense form of the disease. In the past, this has involved extensive clearance of bush to destroy tsetse fly breeding and resting sites, and widespread application of insecticides. More recently, efficient traps and screens have been developed that, usually with community participation, can keep tsetse populations at low levels in a cost-effective manner (Kabayo, 2002; Torr, *et al.*, 2005).

1.2.4 Drug resistance

Since there is no indication that new products against trypanosomaiasis will become available in the near future, it is importance that measures are taken to avoid or delay the development or spread of resistance, and to maintain the efficacy of the currently available drugs. Resistance to one or more of the trypanocidal drugs used in human and cattle has been reported in many countries in sub-Saharan Africa (Peregrine, 1994). This is probably an underestimation of the true situation, because in several countries surveys for resistance have not yet been carried out or cases of resistance have not been published.

Over the past few years, reports of treatment failure in melarsoprol-treated patients are on the increase, particularly in Sudan, Angola, Democratic Republic of the Congo and Uganda. In Uganda, relapse rates of up to 30% have been reported (Legros, *et al.*, 1999). The development of resistance to isometamidium chloride has been reported in both east and west Africa (Peregrine, 1994). The use of a double dose of diminazene only slightly improved the therapeutic efficacy for resistant *T. congolense* (Silayo, *et al.*, 1992). The weekly monitoring of cattle and treatment with diaminazine aceturate failed to provide effective control and as a result, almost a quarter of the animals died or removed from the trial in an extremely weak condition (Stevenson, *et al.*, 1995). *T.*

brucei was resistant to diminazene and isometamidium in Nigeria (Kalu, 1995), and in Kenya resistance to melarsoprol has been reported in *Trypanosoma brucei rhodesiense* (Bales, *et al*, 1989). In Uganda, *Trypanosoma brucei rhodesiense were* resistant to melarsoprol (Ogada, 1974), and *T. brucei* to diminazene and isometamidium (Matovu, *et al.,* 1997), and *Trypanosoma brucei rhodesiense* to DFMO (Iten, *et al.,* 1995). Resistance has also been found in *T. brucei* against isometamidium in Zambia (Chitambo and Arakawa, 1991), and, in D.R.Congo, *Trypanosoma brucei gambiense* was resistant to melarsoprol (Pepin, *et al.,* 1987). Resistance to one drug may also occur as a result of induction of parasite resistance to other related compounds (Mwambu, and Mayende, 1971). Such cross-resistance to diminazene, isometamidium and homidium, for example, is thought to develop as a result of resistance to quinapyramine (Ndoutamia, *et al.*, 1993)

1.3 Toxoplasmosis

1.3.1 Disease

Toxoplasmosis is a disease caused by an intracellular protozoan parasite, *Toxoplasma gondii*, and is widespread throughout the world (Jackson and Hutchison, 1989; Bhopale, *et al.*, 1997). *Toxoplasma gondii* has very low host specificity, and it will probably infect almost any mammal. The disease is of major medical and veterinary importance, being a cause of congenital disease and abortion in humans and in domestic animals (Joiner and, Dubremetz, 1993). Humans can be infected with *T gondii* by ingestion or handling of undercooked or raw meat (mainly pork and lamb) containing tissue cysts or water or food containing oocysts excreted in the faeces of infected cats. In addition to being swallowed, *Toxoplasma* parasites can enter the body through contaminated blood transfusions or through organ transplants taken from infected donors. The United States Department of Agriculture (USDA) estimates that 50% of *T. gondii* infections in the United States are caused by ingestion of raw or undercooked infected meat (Buzby and Roberts, 1996). This was confirned by a community-based study in Maryland (Roghmann, *et al.*, 1999). In 1999, Mead and colleagues estimated deaths caused by toxoplasmosis at 750 each year, making toxoplasmosis the third leading cause of foodborne deaths in the US (Mead, *et al.*, 1999). According to the U.S. Centers for Disease Control and Prevention (CDC), an estimated 225,000 cases of toxoplasmosis occur every year in the United States, with 5,000 hospitalizations and 750 deaths. About 50% of all cases of toxoplasmosis are related to food. In the United States, up to 4% of lamb products and 32% of pork products are contaminated with toxoplasmosis. In some areas of Brazil, the serological prevalence of *T. gondii* infection ranges from 50% to 80% of the adult population, with the highest values found in some northern and southern states (Bahia-Oliveira, *et al.*, 2003). In northern Rio de Janeiro State, water has been identified as a source of *T. gondii* infection (Benenson, *et al.*, 1982; Bowie, *et al.*, 1997).

The disease is commonly asymptomatic when acquired by healthy persons. However, it can cause significant morbidity and mortality in people whose immune defenses are weakened because of AIDS, cancer or immunosuppressant medication. Under these coditions, a new toxoplasmosis infection may spread out of control and become deadly, or dormant *Toxoplasma* parasites from an old toxoplasmosis infection may suddenly become active again and cause severe illness. This situation is especially dangerous for people with AIDS. In these people, dormant toxoplasmosis can reactivate and cause a severe brain infection (encephalitis), which can lead to seizures and other neurological problems (Araujo and Remington, 1987; Luft and Remington, 1992; Jones, *et al.*, 1999). If left untreated, the death rate from encephalitis is very high.

Also, if a toxoplasmosis infection develops in a pregnant woman, there is a 50% chance that the parasites will cross the placenta and cause toxoplasmosis in the infant, resulting in a spontaneous abortion, a still born, or a child that is severely handicapped mentally and/or physically. This is called congenital toxoplasmosis (Remington and Desmont, 1973). In the United States, congenital toxoplasmosis occurs in 400 to 4,000 newborns each year (CDC. Report, 2000), and these newborns are at high risk of toxoplasmosis-related eye problems and developmental disabilities (Jones, *et al.*, 2001).

Toxoplasmosis is causing great financial loss in the agricultural industry (Schwartzman, 2001), being a major cause of abortion and stillbirth in sheep flocks in Britain, Australia and New Zealand. Toxoplasmosis is the most frequently diagnosed cause of abortion in sheep and accounts for approximately 80% of all sheep abortions in the UK. Abortions are higher in colder areas, and while 30% of ewes in a flock aborting would be considered at the higher end of the scale it is not unknown. Toxoplasmosis occurs in isolated occurrences in Tasmania throughout the lambing season. While the economic impact to the industry is small, high lambing loss percentages can have a devastating financial impact on individual farm businesses in any one season. Reports suggested that some farm businesses no longer run ewes and lambs because of toxoplasmosis

(Tasmanian Farmers & Graziers Association report TFGA. Report, 2004). In the U.S, pigs are also infected with an estimated 3% of market pigs and 18% of breeding stock. The parasitic infection persists in pigs despite good quality management procedures. Among food animals, pigs are the major source of human infection. Domesticated pigs can become infected by eating feed contaminated by cats, or by eating other infected hosts such as dead rodents in hog pens (College of Agricultural, Food, and Environmental Sciences. CAFES. Report, 2001). In the UK, about 0.5-1% of individuals become infected each year.

The most common symptoms of the infection are confusion, headaches, fever, paralysis, seizures, and difficulty seeing, speaking and walking. Untreated, the infection can lead to progressive lethargy (lack of energy), coma and even death. The most important factors in preventing the complications of toxoplasmosis are early recognition of symptoms, prompt initiation of treatment, and lifelong suppressive therapy to prevent the recurrence of brain abscesses.

1.3.2 Organisms and life cycle

Toxoplasma gondii is an obligate intracellular protozoan parasite that belongs to the phylum Apicomplexa, class Sporozoa, subclass Coccidian, and suborder Eimeria (Levine, *et al.*, 1980). It is therefore related to malaria and a number of coccidians that generally infect birds and most mammals, including man (Joiner and Dubremetz, 1993). The parasite was discovered independently by the French parasitologists Charles Nicolle and Louis Herbert Manceaux while looking for a reservoir host of *Leishmania* in a North African rodent, the gundi *Ctenodactylus gondii* (Schwartzman, 2001). *T. gondii* is a single-celled parasitic organism that can infect most animals and birds, but because it reproduces sexually only in cats, wild and domestic felines are the parasite's ultimate host (Figure 1.9).

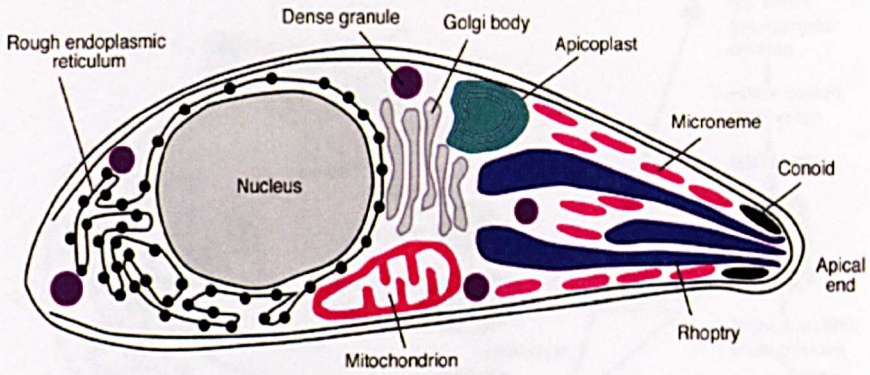


FIGURE 1.9 Diagrammatic representation of the ultrastructure of a *Toxoplasma gondii* tachyzoite. Reproduced from Ajioka, *et al.* (2001).

T. gondii's complex life cycle begins when a cat eats infected prey, usually a mouse or bird. Cats can also become infected if they are fed raw, contaminated meat or ingest infected soil. Once ingested, *T. gondii* burrows into the walls of the cat's small intestine, forming early-stage cells called oocysts that the cat eliminates in its feces, usually for a period of two to three weeks. Oocysts, which are shed in feces of recently infected cats, are resistant to desiccation and heat (Dubey, 1995). A single stool may contain millions of oocysts. Most healthy cats will not shed oocysts after this initial acute stage. Within a few days, the oocysts develop into mature, highly-infectious cells that under certain conditions can survive in the soil for a long time. When they are ingested by another animal, they multiply rapidly inside the host, eventually forming inactive eggs (cysts) that lodge mainly in the brain or muscles (Figure 1.10). The new host animal usually remains symptom-free and will not excrete oocysts (Tenter, *et al.*, 2000).

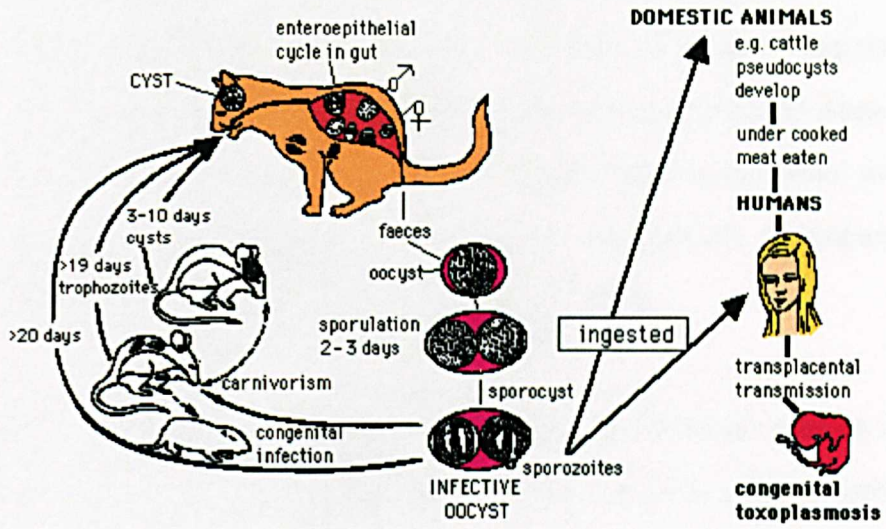


FIGURE 1.10 Diagrammatic representation of the life cycle of *Toxoplasma gondii.* Obtained from: Protozoa as Human Parasites. http : // www-micro .msb .le.ac.uk / 224 / Parasitology.html.

In many respects, the pattern is similar in humans, after infection with *T. gondii,* the parasite forms cysts that can affect almost any organ but most often affects the brain, skeletal and heart muscle. If the person is generally healthy, the immune system keeps the parasites in the body in an inactive state for life. This provides immunity so that the person can not become infected with the parasite again. However, if the resistance is weakened by disease or certain medications, the infection can be reactivated, leading to serious complications.

1.3.3 Therapy

Most healthy people do not require any treatment for toxoplasmosis, unless having signs and symptoms of acute toxoplasmosis. The major drugs used for the therapy of toxoplasmosis are targeted at the folate pathways of the parasite. The mainstay, and currently most effective therapy for toxoplasmosis, is the combination of

pyrimethamine (Daraprim) and sulfadiazine, which has been used for both adults and children. Using pyrimethamine may prevent your body from absorbing the important B vitamin folate (folic acid, vitamin B-9), especially when taking high doses over a long period of time, it may recommend taking additional folic acid. Other potential side effects of pyrimethamine include bone marrow suppression, leukopenia, anemia, thrombocytopenia and liver toxicity (Schwartzman, 2001).

Sulfadiazine is the preferred sulfonamide, which is an antibiotic used in combination with pyrimethamine to treat toxoplasmosis. Possible side effects include nausea, vomiting and diarrhea. In pregnancy, spiramycin is usually given and it has not been shown to be teratogenic, and appears to decrease the severity of disease (Hohlfeld, *et al.,* 1989; Montoya and Liesenfeld, 2004). This drug has been extensively used in Europe.

Clindamycin, a lincomycin, inhibits the parasite by unknown mechanism that involves the parasite organelle called the apicoplast (Fichera and Roos, 1997). Clindamycin is often substituted for sulfadiazine in patients with sulfa allergy (Gabriel Torres, 1991). One retrospective study showed that 11 of 15 patients treated with clindamycin had clinical improvement and resolution of brain lesions caused by toxoplasmosis (Dannemann, *et al.,* 1988), however, the side effects of clindamycin often preclude its use as a chronic therapy, and toxicities include diarrhea, skin rashes, and abdominal pain. Clindamycin has also been associated with the development of colitis (inflammation of the colon from an overgrowth of bacteria) and strong diarrhea.

Clarithromycin (Klacid) is a macrolide antibiotic which has been shown to inhibit the growth of *Toxoplasma gondii in vitro.* In studies using the blood cells of mice, clarithromycin eradicated infection in 42% of the cells with disseminated lethal toxoplasmosis (Chang, *et al.,* 1988). In a human trial, twelve patients received clarithromycin and pyrimethamine, 75% of them showed total or partial resolution of

brain lesions and a striking clinical response after six weeks of treatment. The high dose of clarithromycin was well-tolerated except for mild hearing loss. Azithromycin is another macrolide antibiotic which has been shown to have potent anti-toxoplasma activity in mice (Araujo, 1988), the drug protected 80% of mice infected with *Toxoplasma.*

Combination regimens are considered to be the first choices for the treatment of different toxoplasmosis. Pyrimethamine, sulfadiazine and leucovorin (folinic acid) are used for the documented fetal infection (after 12 or 18 weeks of gestation), whereas the same combination plus corticosteroids (prednisone) is effective against congenital toxoplasma infection in the infant and toxoplasmic chorioretinitis in adults (Montoya and Liesenfeld, 2004; Bonfioli and Orefice, 2005). The Standard regimens for acute/primary treatment of toxoplasma encephalitis in patients with AIDS include pyrimethamine, leucovorin and Sulfadiazine or Clindamycin. Possible alternative regimens trimethoprim, pyrimethamine and leucovorin plus one of the following: clarithromycin, atovaquone, azithromycin or dapsone (Montoya and Liesenfeld, 2004; Bonfioli and Orefice, 2005).

One group of Spanish researchers reported that 16% of patients, in that study, died within the first week of treatment for toxoplasmosis, regardless of treatment regimen. 50% of the patients who recovered after 21 days of treatment with pyrimethamine and sulfadiazine chose not to continue maintenance therapy and relapsed after an average of 12 months (Pedrol, 1990). None of the patients on maintenance therapy (twice weekly pyrimethamine and sulfadiazine) relapsed after an average follow-up period of 10 months. However, 40% of patients on pyrimethamine and clindamycin maintenance therapy relapsed in this study. A similar study conducted in France reported that two of six patients relapsed on the same regimen (Leport, *et al.,* 1989). Other drugs that have activity against *T. gondii* include dapsone, azithromycine, clarithromycine,

roxithromycine, atovaquone, minocycline and rifabutin (Montoya and Remington, 2000).

1.3.4 Drug resistance

There is very little or almost no data available to address the extent to which drug resistance or strain sensitivity contributes to treatment failures for toxoplasmosis. However, it has been reported that relapse rates, over a long term therapy for toxoplasmic encephalitis in patients with AIDS, were 11% for pyrimethamine-sulfadiazine and 22% for pyrimethamine-clindamycin (Katlama, *et al.*, 1996). It is not known if that was due to the drug resistant. A resistant cell line has also been generated to clindamycin *in vitro* (Tomavo and Boothroyd, 1995). Studies on drugs that are in use in patients, or that show some promise for such use, or that represent lead compounds for further development showed that no instance has yet been reported where resistance to any of these drugs has arisen in a patient or in the field although different strains do show varying degrees of sensitivity (McFadden, *et al.*, 2001). The study of drug resistance generated *in vitro* is an important topic which can provide key information about the drug target. These targets range from metabolic pathways in the cytosol to organellar functions encoded in the mitochondrion or plastid. Such information makes predictions about how fast resistance will arise in the field but more importantly, it helps identify targets that are crucial to the parasite and predicts which combinations of drugs should give good results. The long-term use of antibiotics is expected to impose strong selection for resistant organisms, and it was reported that a single-point mutations in *T. gondii* can produce moderate levels of drug resistance to Pyrimethaminein RH strain parasites (Reynolds, *et al.*, 2001).

1.4 New drug development

At the present time, chemotherapy is still the mainstay to control most parasitic diseases such as leishmaniasis and trypanosomiasis, since antiparasitic vaccines are not yet available against these infections (Berman, 1997; Murray, 2001). There is currently an urgent requirement for new drugs effective against parasitic and bacterial diseases. In developing countries, cases of parasitic diseases such as Leishmaniasis reach 12 million annually. It is important to continue to search for new antiprotozoan molecules because, for some serious parasitic infections, such as leishmania and trypanosomes resistance has developed to the presently available drugs. There is also a need for a new treatment for patients with leishmaniasis co-infections, particularly those with AIDS, since there is a high incidence of relapse after treatment with the current drugs (Desjeux, 1999; Laguna, *et al.,* 1999; Ramos, *et al.*, 1994). Moreover, the treatment of pregnant women, particularly during the first trimester, is often impossible and there is a lack of forms easily usable in children. A better knowledge of the metabolic pathways of protozoa (particularly the apicoplast of Apicomplexan parasites) would certainly open the posssibility to identify new drugs. To reduce and delay the appearance of resistances, mass-treatments of populations should be avoided and targeted treatments prefered, as well as the use of combinations of molecules having different modes of action. The current drugs are, for these reasons, far from being satisfactory drugs and identifying suitable potential drug targets is essential for effective drug development (Smith, 2003).

However, despite the wealth of knowledge generated at the basic research level, drug development against these diseases is still a serious problem as most of the deadly parasitic diseases are distributed in poor countries where is no purchasing power. As such, there is no financial incentive for drug companies to develop the drugs. The development process itself is extremely expensive, so most scientific research stops at the publication stage or falls through the gaps at different stages of the process of the

drug development pipeline (Figure 1.11). Many people and organisations around the world came together to create the Drugs for Neglected Diseases Initiative (DNDi), which is a not-for-profit organisation designed to mobilise resources for Research and Development (R&D) for these diseases (Pecoul, 2004).

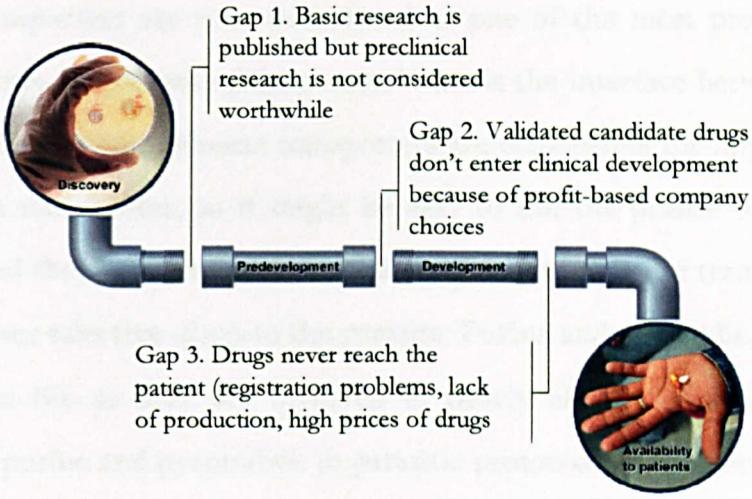


FIGURE 1.11 Diagrammatic representation of the Drug Development Pipeline: Reproduced from Pecoul, (2004).

The fact that the cell biology of protozoan parasites and mammalian cells differ considerably and this distinctness extends to the biochemical level, can provide the promise the potential drug targets. Chemical or genetic manipulation methods to validate these drug targets are now available (Barrett, *et al.,* 1999). In some parasitic protozoa such as leishmania and trypanosomes, glucose metabolism is considered a good chemotherapeutic target, due to the fact that the glycolytic enzymes have structural differences compared with their mammalian counterparts (Aronov, *et al.,* 1999; Verlinde, *et al.,* 2001). The enzymes involved in trypanothione metabolism have also been reported to be a promising drug targets for rational approach to drug design for these parasites (Schmidt and Krauth-Siegel, 2002; Fries and Fairlamb, 2003). Cysteine protease inhibitors have also shown to be effective against some species of

trypanosomes and leishmania *in vivo* and *in vitro*, and they are now undergoing clinical trails against Chagas disease (Sajid and McKerrow, 2002; Troeberg, *et al.*, 1999). Inhibitors of protein kinases have been discovered and appeared to be a potential drug targets as cyclic-dependent kinases are essential to these parasites. These inhibitors have showed good activity against amastigotes in macrophages (Doerig, *et al.*, 2002).

Membrane transporters are now considered as one of the most promising potential drug targets, since the parasite plasma membrane is the interface between the parasite and the host. The transmembrane transporters are responsible for importing nutrients as well as host metabolites, so it might be easy to kill the prasite by blocking these transporters and they could represent good drug targets. Specific transporters can also be used to deliver selective drugs to the parasite. Purine and pyrimidine nucleotides are fundamental to life as they are involved in nearly all biochemical processes. The metabolism of purine and pyrimidine in parasitic protozoa has been studied for the last thirty years. The results of these studies showed that mammalian cells can synthesize the purine ring *de novo* from simple precursors. However, protozoan parasites are incapable of synthesizing the purine ring *de novo* and so must salvage preformed purines from their hosts (Hassan and Coombs, 1988). Furthermore, purine transporters are of considerable pharmacological importance, because both purine analogues and non-purine analogues drugs are taken up by some of these transporters, and loss of permease function can lead to drug resistance (Baldwin, *et al.*, 1999; De Koning and Jarvis, 1999).

1.5 The ENT and CNT nucleoside transporters families

The plasma membranes of all types of cells are the barrier between the organisms and their environment. Membrane transporters are responsible for the uptake of essential nutrients, modulation of concentrations of physiologically relevant chemicals, and

active release of substances such as signaling molecules and waste products. Passive transport is a means of moving biochemicals, and other atomic or molecular substances, across membranes. Unlike active transport, this process does not involve chemical energy. Passive transport is dependent on the permeability of the cell membrane, which, in turn, is dependent on the organization and characteristics of the membrane lipids and proteins. The four main kind of passive transport are diffusion, facilitated diffusion, filtration and osmosis. Diffusion is the net movement of material from an area of high concentration of that material to an area with lower concentration. Facilitated diffusion is movement of molecules across the cell membrane via special transport proteins that are embedded within the cellular membrane. Filtration is movement of water and solute molecules across the cell membrane due to hydrostatic pressure generated by the cardiovascular system. Osmosis is the diffusion of a solvent across a membrane to a region of higher solute concentration.

Mediated transport is another type of transport mechanism. In this mechanism the transport requires the assistance of a transport protein in the membrane to cross the lipid biolayer (Muller and Baker, 1990). Membrane transporters are integral membrane proteins that mediate the facilitative or active transport requirements across membranes and they are often highly selective about the chemicals they allow to cross. In the case of active transport, the proteins are capable of transporting their substrates against the concentration gradient. One example is the sodium-potassium pump in nerve cells: Na^+ is maintained at low concentrations inside the cell and K^+ is at higher concentrations. The reverse is the case on the outside of the cell (Campbell and Reece, 2004).

Natural nucleosides and nucleobases are very important metabolites and have a vast number of physiological roles in many organs, systems and species. The uptake of nucleosides or nucleobases is essential for nucleic acid synthesis in many human cell types and in parasitic organisms that cannot synthesize nucleotides *de novo*. The

transporters responsible are also the route of entry for cytotoxic nucleoside or nucleobase analogues for treatment of many diseases such as parasitic infections, cancer and viral chemotherapy.

There are two main classes of transport proteins that mediate the movement of nucleosides and free purine and pyrimidine bases across eukaryotic plasma membranes. These two unique families are non-homologous and classified by their mechanism of translocation of substrate. The first family is the equilibrative nucleoside transporter (ENT) family that allows the movement of these molecules along concentration gradients into or out of cells (Baldwin, *et al.,* 2004). The second family is the concentrative nucleoside transporter (CNT) family, which actively transport nucleosides into cells by coupling their transport to the inward movement of sodium ions (Gray, *et al.,* 2004). The identification and classification of these families has been based primarily on sequence homology. The equilibrative nucleoside transporters (ENT) family and the concentrative nucleoside transporters (CNT) family were thought to transport primarily nucleosides (Baldwin, *et al.,* 1999). However, with increasing knowledge of the ENT family, it has clearly become that ENT members can transport nucleobases as well (Hyde, *et al.,* 2001; De Koning, *et al.*, 2005).

The concentrative nucleoside transporters are found in many different mammalian tissues such as in intestine, kidney and liver (Baldwin, et al., 1999). The first members of this family were probably identified about 30 years ago, when Strauss and colleagues described the uptake of thymidine and adenosine by murine bulk nonadherent spleen cells. The results showed that the two uptake systems are markedly different (Strauss, et al., 1976). Since that time, a lot of work has been done to identify and characterize many members of this family. The most recent name and classification was given to this family was (SLC28) which consists of three subtypes of sodium-dependent concentrative nucleoside transporters, SLC28A1, SLC28A2 and SLC28A3 which used to be named as CNT1, CNT2 and CNT3, respectively (Gray, *et al.*, 2004). CNT1 or cit-type

transporters (Concentrative, Insensitive to nitrobenzylthioinosine NBMPR and accepts Thymidine as permeant) are specific for pyrimidine nucleosides and were identified in rat liver tissue (Huang, et al., 1994). A similar transporter was identified in human intestine and called hCNT1 (Ritzel, et al, 1997). The CNT2 or cif-class transporters (Concentrative, Insensitive to nitrobenzylthioinosine NBMPR and accepts Formycin B as permeant) are specific for purine nucleosides and uridine, were also found in rat intestine by cloning and called rCNT2, or sodium-linked purine nucleoside transporter SPNT (Ritzel, et al., 1998; Yao, et al., 1996). A similar transporter has been identified in human kidney and designated hCNT2 (Che, et al., 1995). The third subtype is CNT3 or cib-type transporters (Concentrative, Insensitive to nitrobenzylthioinosine NBMPR and accepts a Broad range of permeants) (Figure 1.12), which is the most recent subtype to be identified by molecular cloning from human (hCNT3) and mouse (mCNT3). CNT3 transports both pyrimidine and purine nucleosides into cells in a sodium-linked manner (Ritzel, et al., 2001b; Cass and Young, 2001). The phylogenetic tree for the CNT family shows three clusters. One includes the NupC proteins of E. coli and B. subtilis, the second includes all mammalian symporters, and the third includes functionally uncharacterized bacterial homologues (Saier, et al., 1999). Although the CNT transporter family is widely distributed in both bacteria and eukaryotes, no members of this family have been found in protozoa.

Equilibrative nucleoside transporters (ENTs) are a family of proteins with no apparent sequence homology to other types of transporters, which transport mainly nucleosides, such as adenosine or nucleoside analogues used in chemotherapy across cell membranes (Hyde, *et al.,* 2001). It is now known that the ENT transporter family are widely distributed in mammals, plants, yeasts, insects, nematodes and protozoa, but absent from prokaryotes. However, a possible homology with outer membrane channels involved in the transport of nucleosides into gram-negative bacteria has been postulated (Acimovic and Coe, 2002).

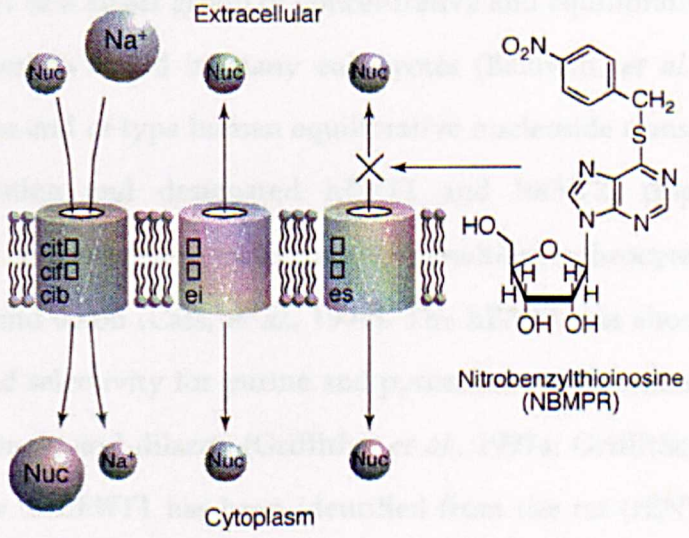


FIGURE 1.12 Diagrammatic representation of the Nucleoside transport systems in mammalian cells. Showing both the equilibrative and concentration nucleoside systems: Reproduced from Baldwin, *et al.* (1999).

The early classification of the ENTs family in mammalian cells was based on their sensitivity to inhibition by nitrobenzylthioinosine (NBMPR), and divided ENTs into Equilibrative Sensitive (*es*) and Equilibrative Insensitive (*ei*) (Figure 1.12). Both systems were found to have broad substrate specificity for purine and pyrimidine nucleosides with a low or moderate affinity (K_m values of ~ 20-400 μM), with different sensitivity to inhibition by NBMPR. Adenosine was found to display the highest affinity for the ENT family members with a K_m of 20-50 μM. However, the affinity of nucleosides for *es*-type transporters, including adenosine, appeared to differ between cell types such as in bovine chromaffin cells (K_m of 1-2 μM). The *es*-type transporter has a high affinity for NBMPR inhibition of nucleoside transport with K_i values of 0.1-10 nM (Plagemann, *et* al., 1988; Griffith and Jarvis, 1996). In contrast to *es*-type, the *ei*-type transporter appeared to be resistant to inhibition by nanomolar concentrations of NBMPR, and in general possess a lower affinity for substrates than the *es*-type transporter.

The SLC29 family, which is the new name of the human equilibrative nucleoside transporters, is part of a larger group of concentrative and equilibrative nucleoside and nucleobase transporters found in many eukaryotes (Baldwin, *et al.,* 2004). The first examples of *es*-type and *ei*-type human equilibrative nucleoside transporters have been identified by cloning and designated hENT1 and hENT2, respectively. hENT1 (SLC29A1) mRNA is expressed in many tissues, including erythrocytes, placenta, brain, heart, liver, lung and colon (Cass, *et al.,* 1999). The hENT1 was shown to be a typical *es*-type, with broad selectivity for purine and pyrimidine nucleosides and inhibited by NBMPR, dipyridamole and dilazep (Griffiths, *et al.,* 1997a; Griffiths, *et al.,* 1997b). A transporter similar to hENT1 has been identified from the rat (rENT), and proved to have similar kinetic properties but it was not inhibited by dipyridamole (Yao, *et al.,* 1997). Another homologue of hENT1 has been identified from a mouse (mENT1). Like hENT1, hENT2 (SLC29A2) mRNA has also been found in many tissues, but is particularly present in skeletal muscle (Crawford, *et al.,* 1998). The hENT2 appeared to be a typical *ei*-type transporter and has similar broad selectivity for purine and pyrimidine nucleosides, and might also transport the purine nucleobase hypoxanthine (Osses, *et al.,* 1996). Homologues to hENT2 have also been identified in the rat and mouse, designated rENT2 and mENT2, respectively. The most recent isoforms have been identified are ENT3 and ENT4. The cloning of cDNAs encoding mouse and human ENT3 has been reported (Hyde, *et al.,* 2001), and it has been suggested that hENT3 is a broad selectivity, low affinity nucleoside transporter that might also transport adenine. In addition, transport activity was relatively insensitive to the nucleoside transport inhibitors nitrobenzylthioinosine, dipyridamole, and dilazep and was sodium ion-independent (Baldwin, *et al.,* 2005). The hENT4 gene was originally identified by genome database analysis and appeared to be a low affinity monoamine, rather than a nucleoside, transporter and has been alternatively designated plasma membrane monoamine transporter (PMAT) (Acimovic and Coe, 2002). It has been confirmed that, by examining the effect of mutating the targeting motif on the subcellular location of hENT3, this transporter is indeed intracellular and partially co-

localizes with lysosomal markers. Relocation of the hENT3 to the cell surface in the absence of the motif has allowed detailed characterization of its functional properties when expressed in *Xenopus* oocytes.

Recently, several studies have tried, by site directed mutagenesis and other techniques, to elucidate the domains of ENT transporters involved in substrate binding and interactions with the standard inhibitors. This was recently reviewed by De Koning et al. (2005). In addition, progress has been made in identifying the functional groups of the substrates that are recognised by the hEN1 and hENT2 binding sites (Vickers, *et al.*, 2002; Vickers, *et al.*, 2004). This studies rrevelealed that both transporters were sensitive to modifications at C(5') and hENT2 displayed more tolerance to removal of C(5')-OH than hENT1; addition of an O-methyl group at C(5') greatly reduced interaction with either hENT1 or hENT2. The changes in binding energies between transporter proteins and the different uridine analogs suggested that hENT1 formed strong interactions with C(3')-OH and moderate interactions with C(2')-OH and C(5')-OH of uridine, whereas hENT2 formed strong interactions with C(3')-OH, weak interactions with C(5')-OH, and no interaction with C(2')-OH.

1.6 Purine transport in protozoa – the role of the ENT family

Transport mechanisms in parasites have been studied for many years as transporters play a central part in the biochemistry of all parasitic protozoa. Transport studies are important to understand how parasites acquire nutrients from their hosts and take up drugs, which are an important determinant of therapeutic efficacy (Landfear, 2000). On the basis of sequence homology, protozoan nucleoside transporters characterised to date have all been classified as belonging to the equilibrative nucleoside transporter (ENT) family, which includes the human equilibrative nucleoside transporters (Hyde, *et al.*, 2001).

Almost nothing was known about the mechanism of nucleobase transport in parasites until the recent studies on trypanosomes when two purine nucleobase transporters were identified in procyclics of *T. brucei brucei.* The first (H1) is a high-affinity transporter specific for purine nucleobases such as hypoxanthine (K_m of 9.3 μM), and it does not transport pyrimidine nucleobases or purine and pyrimidine nucleosides (De Koning and Jarvis, 1997a). The second purine nucleobase transporter, which was identified and characterized in our laboratory and cloned by Richard Burchmore and designated TbNBT1 or H4, transports all natural purine nucleobases, with high affinity for adenine and hypoxanthine (K_m values of 2.1 and 0.66 μM, respectively), as well as uracil and allopurinol (Burchmore, *et al.*, 2003; Chapter five). The bloodstream form has also two purine nucleobase transporters: H2 which displays a very high affinity for all natural purine nucleobases, such as hypoxanthine (K_m of 123 nM), and their analogues such as allopurinol, as well as for guanosine. The second transporter expressed in bloodstream forms, H3 (De Koning and Jarvis, 1997b), is very similar to H1 with respect to purine nucleobases (K_m value of 4.7 μM for hypoxanthine).

An independent study has identified a very similar transporter to TbNBT1, designated TbNT8.1 which was functionally expressed in *Xenopus oocytes* and nucleoside-nucleobase transport-deficient yeast (Henriques, *et al.,* 2003). This transporter possess a high affinity for all natural purine nucleobases K_m values of 3.1 μM, 8.0 μM, 12.4 μM, and 28.5 μM for Hypoxanthine, adenine, guanine, and xanthine, respectively. A number of very closely copies of genes to *TbNT8.1* and *TbNBT1,* have also been identified and designated *TbNT8.2* and *TbNT8.3* (De Koning, *et al.,* 2005).

The strict selectivity for purines (but not for uric acid) sets the nucleobase transporters in parasitic protozoa clearly apart from those in fungi of mammalian cells (De Koning and Diallinas, 2000). *T. b. brucei* thus express multiple nucleobase transporters, both in procyclic and in bloodstream forms, each with high affinity for allopurinol (De Koning and Jarvis, 1997a; De Koning and Jarvis, 1997b; Burchmore, *et al.,* 2003). This

observation is believed to be the reason allopurinol-resistance is very difficult to induce in trypanosomes (Natto, *et al.*, 2005).

Nucleobase transporters in protozoa usually display a higher affinity for their substrates than do mammalian transporters. One well-characterised example is the *T. brucei* H2 transporter, when compared to the equilibrative nucleobase transporter of human erythrocytes hRBC. The basis for the different affinities was shown to be the result of very different interactions of the respective transporters with the purine ring (Wallace, *et al.*, 2002). In contrast to mammalian cells, the *T. brucei brucei* purine nucleobase transporters, like their nucleoside transporters (De Koning, *et al.*, 1998; De Koning and Jarvis, 1999; De Koning, *et al.*, 2000b), are not inhibited by either nitrobenzylthioinosine NBMPR, papaverine, dilazep, or dipyridamole and are not sodium ion-dependent transporters (De Koning and Jarvis, 1997a; De Koning and Jarvis, 1997b). H1 and H2 are nucleobase/proton symporter as they are linked to a protonmotive force (De Koning and Jarvis, 1997a; De Koning and Jarvis, 1997b), while the energy-dependence of H3 is still unclear. The differences in affinities of parasite and human nucleobase transporters could help in the design of chemotherapeutic purine analogues that are selectively transported by the parasites.

An early study has demonstrated the transport of purine nucleosides adenosine, inosine and guanosine by *Trypanosoma congolense* and *Trypanosoma brucei*. In that report, it was also demonstrated that the purine nucleobases hypoxanthine and adenine were taken up by the same parasites. In both species adenosine strongly inhibited the uptake of both of the other nucleosides and of both purine bases. In addition, the uptake of adenosine by *T. brucei* was inhibited by dipyridamole (James and Born, 1980).

Two classes of nucleoside transporter genes have been identified in *Trypanosoma brucei*, TbNT2 and TbAT1, encoding transport activities known as P1 and P2, respectively. Both are high-affinity adenosine transporters with K_m values of 0.15 and

0.55 μM for P1 nd P2, respectively, (Carter and Fairlamb, 1993; De Koning, *et al.*, 1998). P1 has broad specificity for purine nucleosides such as inosine (K_m = 0.36 μM) and guanosine (K_i = 0.36 μM) and it has very low affinity for purine nucleobases such as adenine and hypoxanthine as well as the pyrimidine nucleoside uridine (K_i > 1 mM (De Koning, *at al.*, 1997b; Sanchez, *et al.*, 1999; De Koning, *at al.*, 2005). The gene encoding this transporter has been cloned by expression in *Xenopus* oocytes and was designated *TbNT2* (Sanchez, *et al.*, 1999). This P1-type transporter is expressed in both bloodstream form (BF) and in procyclic form (PF) trypanosomes, and the P1 transporter was the first protozoan nucleoside transporter shown to be a proton symporter (De Koning, *et al.*, 1998). P2 is the second nucleoside transporter and, in contrast with P1, this transporter displays high affinity for aminopurines such as adenine (K_i = 0.38 μM) and adenosine, but has low affinity for oxopurines such as inosine, guanosine, hypoxanthine, guanine and allopurinol (Carter and Fairlamb, 1993; Mäser, *et al.*, 1999; De Koning and Jarvis, 1999; De Koning, *at al.*, 2005). This transporter is expressed only in the BF stage of the life cycle. The *TbAT1*, the gene encoding the P2 transporter, was cloned by functional complementation in *Saccharomyces cerevisiae*, which was naturally deficient in purine nucleoside uptake (Mäser, *et al.*, 1999). Since P2 can mediate the uptake of antitrypanosomiasis diamidines, such as pentamidine and berenil (diminazene aceturate), and melaminophenyl arsenical, this transporter has become of a particular pharmacological interest (Carter, *et al.*, 1995; De Koning, *et al.*, 2000a; De Koning and Jarvis, 2001). De Koning and colleagues have studied the substrate recognition motifs of adenosine transporters in bloodstream form *T. brucei* and reported that the predicted substrate recognition motif of P2, but not of P1, corresponds to parts of the melaminophenylarsenical and diamidine molecules, explaining the potent inhibition observed with these trypanocides for P2-mediated adenosine transport (De Koning and Jarvis, 1999). P1 and P2 systems have also been characterised in different species of trypanosomes such as *T. equiperdum* (Barrett, *et al.*, 1995), *T. evansi* (Suswam, *et al.*, 2001).

A cluster of six genes, designated *TbNT2* to *TbNT7* has been reported (Sanchez, *et al.*, 2002). The ORFs of these genes were about 85% identical to that of TbNT2. Functional expression in *Xenopus oocytes* showed that TbNT2, TbNT5, TbNT6 and TbNT7 all have high affinity for purine nucleosides such as adenosine, inosine and guanosine with K_m values of ~ 1 μM). However, TbNT5, TbNT6, and TbNT7 also transported the purine nucleobase hypoxanthine all with much lower affinity (K_m = ~ 25 μM). TbNT3 and TbNT4 did not appear to transport nucleosides or nucleobases and their function is still unclear. mRNAs from all genes in the these transporters were expressed in BF, but TbNT2, and TbNT5 mRNAs were also found in PF. It has been suggested that different members of this transporter family have different substrate specificities and are expressed in different stage in the life cycle (Sanchez, *et al.*, 2002). Further members have been identified from the ENT family by examination of *T. brucei* database and have been named as TbNT8, TbNT9, TbNT10 and TbNT11. TbNT8 is thought to represent a family of clustered genes (Landfear, *et al.*, 2004), TbNT8.1 is one member of this family and it has been discussed in the nucleobase transporter section. It has been reported that TbNT10 is a high affinity purine nucleoside transporter. Functional expression in *Saccharomyces cerevisiae* revealed a high-affinity nucleoside transporter for adenosine, inosine, and guanosine with K_m values of about 1 μM. Its mRNA was found to be expressed exclusively in the nondividing short stumpy stage (Sanchez, *et al.*, 2004a). It has also been reported that TbNT11 transporters are represented by two closely related genes, TbNT11.1 and TbNT11.2, but they have not been functionally characterised yet.

An important difference between purine nucleoside transporters in trypanosomes and their counterparts in mammalian cells is that they are protonmotive force-driven transporters and are not inhibited by NBMPR, dilazep, or dipyridamole at the concentration required inhibiting NBMPR-sensitive mammalian nucleoside transporters (De Koning, *et al.*, 1998; De Koning and Jarvis, 1999). Furthermore, the trypanosome nucleoside transporters, especially TbNT1, have high affinity for toxic

purine nucleoside analogues (e.g., tubercidin, ribavirin, and Formycin A), which are poor ligands for the mammalian transporters (De Koning and Jarvis, 1999).

In general, there is a very little known about purine nucleobase transporters and their kinetic properties in *leishmania* species. The first purine nucleobase transporters have been identified in *Leishmania braziliensis panamaensis* (Hansen, *et al.,* 1982). This report suggested that three transporters could be identified in promastigote forms of *Leishmania braziliensis.* The first transporter, designated Locus 1, transported inosine, Locus 2, the purine bases hypoxanthine and adenine and Locus 3, adenosine. The same group found that adenine and hypoxanthine inhibited the uptake of one another competitively which suggests that they are using the same transporter (Hansen, *et al.,* 1982).

We have conducted a comprehensive study of purine nucleobase uptake in Leishmania major promastigotes. We have identified and characterized in great detail a single transporter, designated LmajNBT1, with high affinity for all physiological purine bases and moderately high affinity for allopurinol. Studies with [^{3}H]allopurinol confirmed that this transporter is its sole route of entry into promastigotes, as no study has addressed allopurinol uptake by this parasite (Chapter three). Sanchez and colleagues cloned and expressed the first gene for a *L. major* nucleobase transporter, designated LmaNT3. The LmaNT3 permease shows 33% identity to the *L. donovani* nucleoside transporter LdNT1.1, and is, thus, a member of the equilibrative nucleoside transporter (ENT) family (Sanchez, *et al.*, 2004b). LmaNT3 is a high affinity transporter for hypoxanthine, xanthine, adenine and guanine, which is very similar to the LmajNBT1 transport activity reported in our study (Chapter three).

We have also investigated nucleobase uptake in *L. mexicana* amastigotes and identified the first nucleobase transporter in *Leishmania* amastigotes, and characterization the transport of allopurinol. Our data are consistent with a single broad specificity

nucleobase transporter mediating the uptake of all natural purine nucleobases and allopurinol. The functional characterization of this transporter, LmexNBT1, was shown to be virtually identical to LmajNBT1. The binding interactions between the purine nucleobases and this transporter were compared with those of the LmajNBT1 transporter in *L. major* promastigotes and found to be very similar (Al Salabi and De Koning, 2005).

Early studies reported that *L. donovani* promastigotes possess two purine nucleoside transporters with nonoverlapping substrate specificity, designated LdNT1 and LdNT2 (Iovannisci, *et al.,* 1984; Aronow, *et al.,* 1987). The first transported adenosine, analogues of adenosine, formycin A and tubercidin and the pyrimidine nucleosides, uridine, cytidine, and thymidine, while the second carried inosine, guanosine, formycin B and their analogues. The two nucleoside transport systems of *L. donovani* were not inhibited by 4-nitrobenzylthioinosine and dipyridamole, two potent inhibitors of nucleoside entry into mammalian cells (Aronow, *et al.,* 1987). However, the transport of adenosine seemed to have different activities in various strains of *L. donovani* (Ogbunude, *et al.,* 1991) and *L. major* (Baer, *et al.,* 1992) promastigotes, though such variations have not been noted in inosine transport. Two closely linked genes have been identified, designated *LdNT1.1* and *LdNT1.2* that encoded the LdNT1.1 and LdNT1.2 transporters which mediated the uptake of adenosine and the pyrimidine nucleosides with high affinity (LdNT1.1; K_m values of 0.17 and 5.6 μM for adenosine and uridine, respectively; LdNT1.2; K_m values of 0.66 and 40 μM for adenosine and uridine, respectively). A single gene encoding the functional inosine-guanosine transporter LdNT2 (K_m values of 0.3 and 1.7 μM for inosine and guanosine, respectively), and has been cloned by functional rescue of the mutant phenotype designated *LdNT2.* These transporters are members of the ENT family as the amino acid sequences and topologies showed substantial homology to other members of the family (Vasudevan, *et al.,* 1998; Carter, *et al.,* 2000b). The nucleoside transporters of *L.*

donovani promastigotes have been shown to be proton-dependent rather than sodium-dependent (Stein, *et al.*, 2003).

Two adenosine transporters have also been reported in *Leishmania donovani* amastigote named as T1 and T2 (Ghosh and Mukherjee, 2000). T1 is adenosine/pyrimidine transporter and was very similar to the LdNT1 activity which was originally detected in promastigotes (K_m values of ~ 1 μM for adenosine). T2, the second adenosine transporter, is inhibitable by inosine and exclusively presents in amastigotes (K_m values of ~ 1 μM for adenosine).

The first study of purine uptake in the apicomplexan parasite *Toxoplasma gondii* was published by Schwab and colleagues. An adenosine transporter (TgAT1) was identified in the plasma membrane of *T. gondii* tachyzoites with a K_m of 230 μM (Schwab, *et al.*, 1995), which has low affinity for the nucleoside. This group described a similar inosine-sensitive adenosine transporter in *T. gondii*. A low affinity transporter with very similar inhibitor profile to this transporter activity was cloned by insertional mutagenesis and selection on a cytotoxic adenosine analogue, adenine arabinoside and designated *TgAT* (Chiang, *et al.*, 1999). These two groups also reported that adenine and hypoxanthine inhibited adenosine, suggesting that this transporter mediated both nucleoside and nucleobase uptake but at a low level. This transporter was inhibited by dipyridamole (IC_{50} = 0.7 μM) but not nitrobenzylthioinosine, and is also a member of the ENT family (De Koning, *et al.*, 2005). We decided to reinvestigate purine transport in *T. gondii* tachyzoites for two reasons. First, because of the fact that *Toxoplasma* is a purine auxotroph and there is no credible source of purines that could be salvaged by the low affinity transporter TgAT1. Second, because *T. gondii* are able to salvage nucleobases efficiently but no high affinity transporter has been described for this organism. We have identified a high affinity hypoxanthine/guanine transporter, which is the first high affinity nucleobase transporter to be identified in an apicomplexan parasite, designated TgNBT1 (K_m for hypoxanthine of 0.91 μM. We have also reported

that *Toxoplasma gondii* tachyzoites possess two adenosine/inosine transporters designated TgAT1 and TgAT2. In contrast to TgAT1, TgAT2 displayed high affinity for all natural pyrimidine and purine nucleosides (K_m values of 0.49 and 0.77 μM for adenosine and inosine, respectively) and binds nucleoside analogues, such as tubercidin (7-deazaadenosine) and AraA, with high affinity (Chapter seven or De Koning, *et al.*, 2003). The genomic database ToxoDB contains complete or nearly complete genomic sequences for *T. gondii* and contains at least three putative homologs of ENT transporters: TgNT1, TgNT2 and TgNT3 (Chaudhary, *et al.*, 2004).

1.7 Rational drug design based on selective transport

The development of new drugs with potential therapeutic applications is one of the most complex and difficult processes in the pharmaceutical industry. Millions of dollars and many of workers are devoted to the discovery of new therapeutical agents. Most of the currently available antiparasitic drugs have been discovered empirically by screening of large numbers of compounds for efficacy against parasites in animal models. Few of these drugs have been rationally designed. This is largely because, until recently, little was known about the basic biochemistry, physiology, and molecular biology of parasites and of their interactions with their hosts.

A major challenge confronting the pharmaceutical scientist is to optimize the selective and efficient delivery of new active entities and drug candidates. Successful drug development requires not only optimization of specific and potent pharmacodynamic activity, but also efficient delivery to the target site. Following advances in rational drug design, combinatorial chemistry and high-throughput screening techniques, the number of newly discovered and promising active compounds has increased dramatically in recent years, often making delivery problems the rate-limiting step in

drug research. To overcome these problems, a good knowledge of the pharmacokinetic barriers encountered by bioactive compounds is required.

The rational design of a drug is usually based on the exploitation of fundamental biochemical and physiological differences between pathogens and their hosts. Some of the most striking differences between parasites and their mammalian host are found in purine metabolism. Therefore, purine metabolism constitutes an excellent potential target for the rational design of antiparasitic chemotherapeutic regimens. Whereas mammalian cells can synthesize the purine heterocycle *de novo*, all protozoan parasites studied to date except for the opportunistic *Acanthamoeba* (Hassan and Coombs, 1988), are purine auxotrophs (Perrotto *et al.*, 1971; Schwartzman and Pfefferkorn, 1982). These parasites lack the ability to synthesize purine nucleotides *de novo*. Instead, they utilize purine salvage pathways to convert the host organism's purine bases and nucleosides to the corresponding nucleotides. As the first step in purine salvage is translocation of the purine across the cell membrane, the transport routes are potential targets for chemotherapy.

Purine and pyrimidine antimetabolites are widely used to combat a variety of infectious diseases and other pathologies. However, many therapies suffer from a lack of selectivity, leading to severe side effects. The selectivity and efficacy of purine antimetabolites is achieved in two ways. Firstly, the enzymes of the purine metabolic pathways that convert the pro-drug to the cytotoxic metabolite, usually a nucleotide analogue (Wang, 1984). Secondly, cell-surface transporters mediate access to the cell, and such as, are potential targets for chemotherapy (Wallace, *et al.*, 2002; Al Salabi, *et al.*, 2003). It has been demonstrated that blocking purine salvage in protozoa through rational design of novel drugs is practicable (Somoza, *et al.*, 1998). A number of groups have also reported on the successful design of enzyme inhibitors directed against trypanosomiasis (Li, *et al.*, 1996; Aronov, *et al.*, 1999; Bressi, *et al.*, 2000; Khan, *et al.*, 2000). Because most purines and their analogs are hydrophilic and diffuse very slowly

across cellular membranes, purine transporters play a primary role in their cellular uptake and release. The uptake of purine nucleosides or nucleobases is essential for nucleic acid synthesis in many human cell types and in parasitic organisms that cannot synthesize nucleotides *de novo*. The transporters responsible are also the route of entry for many cytotoxic nucleoside analogues used in cancer and viral chemotherapy.

Many purine analogues have shown antiprotozoal activity, but no rational approach to a purine-based chemotherapy for protozoan infections has been developed. Such an approach needs to take account of the efficient uptake of the analogue by the parasite and the conversion by the parasite's metabolism to a form harmful to the organism. Selective toxicity therefore needs to be the result of either selective uptake or differences in metabolic enzymes or a combination of the two. Understanding the substrate selectivity of the transporters is critical for the development of new therapeutic purine analogues with optimal pharmacokinetic properties. The utility of many current drugs is limited by insufficient delivery to the target cells. To date, these clinical efforts have failed. We believe that this is due to the fact that these compounds have been non-selective inhibitors of multiple drug transport proteins.

Rationally designed chemotherapeutic agents are usually optimised at the level of the intracellular target. Yet, the efficacy of the drug is in large part dependent on the speed at which it enters the target cell, just as the specificity of the drug and side effects are linked to specific accumulation by the target cell rather than host cells. Specific uptake by a target cell requires intimate knowledge of plasma membrane transporters for the class of drugs being developed. We have modelled the transporter-substrate interactions of purine transporters and shown that these can predict the binding energy of potential substrates by using the transporter kinetics and purine analogues. We have shown that protozoan and human nucleobase transporters interact with their substrates in a very different way, which can be exploited in the rational design of purine-based chemotherapy (Wallace, *et al.*, 2002).

Another reason which makes us interested in the study of purine transporters in protozoa is that these transporters have been implicated in the uptake of trypanocidal drugs such as the case of purine nucleoside transporter in *Trypanosoma* species, and changes to these carriers can lead to drug resistance in *Trypanosoma equiperdum* (Barrett, *et al.,* 1995), *Trypanosoma brucei* (Carter and Fairlamb, 1993; Carter, *et al.,* 1995; Carter, *et al.,* 1999), and *Trypanosoma evansi* (Ross and Barns, 1996). For example, pentamidine is poorly membrane-permeable and active transporters have been described in *Leishmania* species and trypanosomes that allow pentamidine to accumulate to high concentrations (Carter, *et al.,* 1995; De Koning and Jarvis, 1999; Basselin, *et al.*, 2002; Bray, *et al.*, 2003). Another example is the antileishmanial agent allopurinol (Das, *et al.,* 2001; Abrishami, *et al.,* 2002; Momeni, *et al.,* 2002), which is a nucleobase analogue that is efficiently taken up by a high-affinity purine transporter in *L. major* promastigotes (Al Salabi, *et al.,* 2003) and *L. mexicana* amastigotes (Al Salabi and De Koning, 2005). A recent study by De Koning's group compared the ability of a new class of tricyclic purine antimetabolites to interact with transporters from human erythrocytes or *Trypanosoma brucei.* The results showed that these compounds display a remarkable selectivity for the parasite's transporters. The adenine analogue showed greater trypanocidal activity than the hypoxanthine or guanine analogues *in vitro* (Wallace, *et al.,* 2004).

1.8 Purine analogues as therapeutic agents

Despite promising leads in the search for new chemotherapeutic agents, there remains an urgent need to develop more effective and less toxic drugs. Purine nucleobases and nucleosides have fundamental roles in many biological processes and as a result, purine antimetabolites are active chemotherapeutic agents for many infectious diseases and other pathologies. To the extent that these analogues disrupt nucleic acid synthesis or structure, they naturally act predominantly on fast-dividing cells such as cancer cells, viruses and protozoan parasites.

Purine analogues have long been a rich source of chemotherapeutic agents. Modified purine nucleosides, as well as their corresponding purine heterobase moieties have provided a plethora of potent antiviral, anticancer and antiparasitic drugs. Nucleobase analogues are being widely used against infectious agents and malignancies (De Koning and Diallinas, 2000). The purine nucleobases offer a distinct advantage over their nucleoside counterparts since they are readily incorporated into the nucleotide pool by phosphoribosyltransferases while, in contrast, nucleosides may first be hydrolyzed in the bloodstream forms of *T. brucei.*

Some purines have been highly successful in antineoplastic and antiviral therapies and multiple lead compounds aginst protozoa have been identified (Kaminsky, *et al.*, 1994; Seley, *et al.*, 1998; Wanner, 2004; Wallace, *et al.*, 2004). The nucleoside analogues 8-methyladenosine and 2-amino-7-[(1,3-dihydroxy-2-propoxy) methyl] purine (S2242) have been reported as promising anti-poxvirus agents (De Clercq and Neyts, 2004). Purine analogues and their combinations have emerged as effective new therapies for chronic lymphocytic leukemia patients (Wendtner, *et al.*, 2004). Ring-expanded nucleosides (RENs) which are analogues of coformycin also carry promise against many viral infections belonging to the families of hepatitis, herpes, and respiratory infections, most notable being the hepatitis B (HBV), hepatitis C (HCV), and the West Nile Viruses (WNV) (Hosmane, 2002).

Unique features of the purine salvage pathway of protozoan parasites constitute the basis for the susceptibility of these genera to several pyrazolopyrimidine analogues of naturally occurring purine bases and nucleosides (Marr and Berens, 1983; Ullman, 1984). The intact parasites efficiently metabolize these analogues to the nucleotide level, whereas mammalian cells are essentially incapable of these metabolic transformations. One of these pyrazolopyrimidines, allopurinol (4-hydroxypyrazolo[3,4]pyrimidine, HPP) (Berens, *et al.*, 1995), a drug that is nontoxic to humans and is widely used in the treatment of hyperuricemia and gout, has

demonstrated significant therapeutic efficacy in patients with either cutaneous leishmaniasis (Martinez, and Marr, 1992) or chronic Chagas disease (Gallerano, *et al.*, 1990).

Recently, series of tricyclic purine analogues showed to display a promising activity *in vitro* against *T. brucei* and appear to be selectively accumulated by the parasite. These tricyclic analogues represent a significant lead against human African trypanosomiasis (Wallace, *et al.*, 2004).

In addition, purine antimetabolites have long been considered as a promising class of compounds for new drug development against African trypanosomiasis (Avila and Avila, 1981; Ogbunude and Ikediobi, 1982; Avila, *et al.*, 1983; Berens, *et al.*, 1984; El Kouni, 2003), and leishmaniasis (Peters, *et al.*, 1980; Kager, *et al.*, 1981; Berman, *et al.*, 1983; Walton, *et al.*, 1983; Morishige, *et al.*, 1995; Becker, *et al.*, 1999). Using purine analogues as potential antiparasitic agents, was also demonstrated in the treatment of malaria (Gati, *et al.*, 1987; Gero, *et al.*, 1988; Gero, *et al.*, 1989) and other parasitic diseases including schistosomiasis (Jaffe, 1975; El Kouni, *et al.*, 1983; El Kouni, *et al.*, 1985; El Kouni, *et al.*, 1987; El Kouni, *et al.*, 1989; El Kouni, 1991), and toxoplasmosis (Luft, 1986; Sarciron, *et al.*, 1998; El Kouni, *et al.*, 1999).

1.9 Project aims

1. Identify and characterize purine nucleobase and nucleoside transporters in protozoan species such as *Trypanosoma*, *Leishmania*, and *Toxoplasma*.

2. Identify the extent to which these are involved in the uptake of anti-parasitic agents such as allopurinol.

3. Test whether these transporters can be used to facilitate the selective targeting of these parastites by other purine antimetabolites and assess theefficacy of such compounds as chemotherapeutic agents.

4. Assess wheteher purine nucleobase and nucleoside transporters activities are different in various life cycle stages.

Chapter Two

MATERIALS AND METHODS

2.1 Organisms and culture conditions

2.1.1 *Leishmania major* promastigotes

Promastigote forms of *Leishmania major* (Friedlin strain) were grown in Homem culture medium (see Appendix I) (Invitrogen, Carlsbad, CA), supplemented with 10% Heat-inactivated Fetal Calf Serum (Gibco) at 25 °C, in a 5% CO2 atmosphere. Cells in mid-log stage of growth were serially passaged after 3 days of growth. Promastigotes were centrifuged at 2500 g for 10 minutes at room temperature, and cells were then washed twice with assay buffer pH 7.3 (see Appendix I). Parasites were re-suspended at ~10^8 cells/ml, and left for 10 minutes before use in uptake assays.

2.1.2 *Leishmania mexicana* amastigotes

The MNYC/BZ/62/M379 strain of *Leishmania mexicana* was provided by Professor Graham Coombs (Glasgow University). Amastigotes were maintained by twice weekly serial passage in Schneider's Drosophila Medium (GibcoBRL), supplemented with 20% Heat-Inactivated Fetal Calf Serum (HI-FCS; Gibco), containing 0.3 % Gentamycine. The pH of the medium is 6.8 when made up; adding 1.1% 1.0 M of HCl gives 5.5 approximately (pH 5.5-6.0 proved to be satisfactory for the growth of amastigotes). Cultures were maintained at 33°C in a CO_2 incubator. Amastigotes were harvested by centrifugation for 10 minutes at 2500 g at 33°C, and washed twice with the CBSS buffer pH 6 (see Appendix I), and re-suspended at ~10^8 cells/ml in the same buffer (Ghosh and Mukherjee, 2000).

2.1.3 *Trypanosoma brucei brucei* procyclics

Procyclic forms of *T. brucei brucei* strain 427 were grown *in vitro* (Brun and Schonenberger, 1979) in SDM-79 medium (see Appendix I), supplemented with 10%

(v/v) fetal calf serum (Gibco), in a 5% CO_2 atmosphere incubator. Cells in mid-logarithmic stage of growth were serially passaged after 2-3 days of growth, typically at a density of 10^8 cells/ml culture medium. Cells were harvested and washed twice by centrifugation at 2500 g for 15 minutes at room temperature in assay buffer pH 7.3, and seeded at 1 or 2×10^8 cells/ml as required for uptake assays.

2.1.4 *Trypanosoma brucei brucei* bloodstream forms *in vitro*

Bloodstream forms of *T. b. brucei* strain 427 were grown in vitro using HMI-9 medium (see Appendix I), supplemented with 20% (v/v) fetal calf serum (Gibco) at 25°C in a 5% CO_2 atmosphere as described by Hirumi and Hirumi (1989). Cells in mid-logarithmic stage of growth were serially passaged about twice a week. Cells were harvested and washed twice by centrifugation at 2500 g for 15 minutes at room temperature in assay buffer pH 7.3, and adjusted at 1 or 2×10^8 cells/ml as required for uptake assays.

2.1.5 *Trypanosoma brucei brucei* bloodstream forms *in vivo*

Bloodstream forms (usually from frozen stocks but sometimes inoculation is with fresh blood from infected mice or rats) of the *T. brucei brucei* strain EATRO 427, TREU 927, TbAT1-/- and its parent line (TbAT1-WT), were intraperitoneally injected in adult female ICR Swiss mice or Wistar rats. Parasitaemia was monitored by daily examination of blood from tail venepuncture. The microscope field diagram from Herbert & Lumsden (1976) was used to estimate the number of total parasites based on number of parasites per microscope field. The blood from infected rats was collected when peak of parasitemia is reached by cardiac puncture under terminal anesthesia by exposure to CO_2. The blood was collected into a tube containing CBSS buffer with 500 units/ml of heparin and centrifuged at 2500 g for 15 minutes at 4°C to separate the different components. The parasite-containing buffy coat layer that forms at the

intersection between the red blood cell and the plasma, was collected and applied to a DE52 (Whatman, Maidstone, United Kingdom) anion-exchange column (Lanham, 1968) which was adjusted exactly at pH 8.0 and washed with about 200 ml of PSG buffer (see Appendix I) at pH 8.0. The trypanosomes from the column were washed twice in assay buffer at 2200 g for 10 minutes at 4°C, and then suspended in the volume required. The cells were usually left at room temperature for 10-15 minuets prior to use in transport experiments.

2.1.6 Isolation of short-stumpy bloodstream forms

Adult female mice (ICR Swiss strain) were immunocompromised with cyclophosphamide (200mg/Kg) by intraperitoneal injection. Mice were injected intraperitoneally (IP) after 24 hours with ~1×10^6 of *Trypanosoma brucei* strain 927 wild type stabilates. The blood was collected from some of the mice after 4 days and then, using air dried blood smear slides for routine Giemsa staining and NAD Diaphorase assay, the levels of stumpy form trypanosomes were checked microscopically. The procedure was repeated after 9 days with the remaining mice, the trypanosomes population should be predominantly stumpy form. The blood was centrifuged at 2500 g for 15 minutes to separate the different components. Bloodstream stumpies from the buffy coat layer were purified by DE52 chromatography (Lanham and Godfrey, 1970).

NAD Diaphorase assay was performed to confirm the presence of stumpy forms. Briefly, reaction solutions were made fresh just before use containing 4 mg of NADH-disodium-hydrate (Sigma-Aldrich), 0.8 ml of 0.1 M phosphate buffer (pH 7.3), and 1.2 ml of water, and Thin air dried blood smears were prepared (using fresh slides for staining or stored for a brief time on a plastic tray in an ice box). The slides were fixed at 4 °C for exactly 5 minutes by pipetting 0.1 M cacodylate buffer (pH 7.2) with 2.5% final concentration of glutaraldehyde, and then were rinsed quickly in distilled water.

Some slides were incubated for about an hour with the reaction solutions and the other without substrate (control) which is the same, but without NADH. The slides were then mounted in glycerol and viewed under oil at × 1000 or by immunofluorescence microscopy.

2.1.7 *Toxoplasma gondii* tachyzoites

The RH strain of *T. gondii* (Pfefferkorn, *et al.*, 1988) was kindly donated by Dr Elmer Pfefferkorn (Dartmouth College, New Hampshire, USA). *T. gondii* tachyzoites were maintained by twice weekly serial passage in African Green Monkey kidney fibroblasts (Vero cells) and grown in vented tissue culture flasks (Greiner, UK) at 37°C in a 5 % CO_2/95 % O_2 humidified incubator. Host cells were removed from tachyzoites by filtration through 47 mm diameter, 3 μm pore-size Nuclepore® polycarbonate filters (Whatman International Ltd, UK). Isolated tachyzoites, containing typically less than 0.1 % host cell contamination as judged by microscopic observation, were washed three times in assay buffer pH 7.3 by centrifugation at 1500 g for 15 min at 4°C. Tachyzoites were counted using a Neubauer haemacytometer (Weber Scientific Ltd, UK) and re-suspended in assay buffer at a density of 1.5 - 2.5 x 10^8 ml^{-1}.

2.1.8 Yeast Strains

The *Saccharomyces cerevisiae* strain MG887-1 (*fcy2–*) used in this project was engineered to be auxotrophic for uracil by Gillissen and colleagues who excised the truncated *URA3* gene with EcoRI and SmaI from pΔura3 (Gillissen, *et al.*, 2000). This strain dose not possess a functional endogenous uptake system for adenine, hypoxanthine, guanine, or cytosine (Horak, 1997). This yeast strain was used as a heterologous expression system for the cloned members of the ENT transporters in our laboratory. The complete *TbNBT1* , AT-like A, AT-like B, or AT-like D genes open reading frame was excised from pGEMTeasy using *Not*I and subcloned into the *Not*I

site of the yeast expression vector pDR195. A uracil auxotrophic *fcy2* mutant MG887-1 was transformed with the resultant constructs (pDR195:TbNBT1, pDR195:AT-like A, pDR195:AT-like B, and pDR195:AT-like D) and selected on uracil-free medium. Transformed yeast was plated onto yeast nitrogen base without ammonium salts and amino acids, supplemented with 4 mM hypoxanthine as the sole nitrogen source. Information about *Saccharomyces cerevisiae* MG887-1 transformants used in this study is described in chapter six; as well as Lynsey Wallace's PhD thesis 2004 and Burchmore *et al* (2003). *S. cerevisiae* MG887-1 was grown on solid YPAD medium plates (see Appendix I) at 30°C and sub-cultured to a fresh plate once a week.

2.1.9 Bacterial Strains

Two bacterial strains of *Escherichia coli* were used in our laboratory for routine cloning and subcloning of the genomic sequences of *T. b. brucei*. JM109 competent cells and TOP10 electrocompetent cells were obtained from Promega or prepared in our laboratory. Preparations of chemically-competent *E. coli* cells (JM109) and electrocompetent *E. coli* cells (TOP10), and transformations of *E. coli* either by Heat-shock method (for JM109 cells) or by Electroporation (for TOP10 cells), were routinely done in our laboratory (see Appendix for the solutions and methods used).

2.2 Materials

2.2.1 Radiolabeled compounds

[2-^{3}H]Adenosine (0.92 TBq/mmol) and [8-^{3}H]hypoxanthine (1.18 TBq/mmol) were purchased from Amersham Pharmacia Biotech, UK. [2, 8-^{3}H]Adenine (1.19 TBq/mmol) was bought from NEN and from PerkinElmer Life Sciences, and [2, 8-^{3}H]inosine (1.23 TBq/mmol) and [G-^{3}H]Allopurinol (1.9 Ci/mmol) were obtained from Moravek Biochemicals, U.S.A.

2.2.2 Purine and pyrimidine nucleobases and nucleosides and analogues

2-Thiouridine was a generous gift from Dr. D. Davis (University of Utah, USA). 7-Deazaadenosine was from Fluka, cytidine and adenine-9-β-D-arabinofuranoside (AraA) were from ICN, and thiopurinol (4-mercapto-1*H*-pyrazolo(3,4-*d*) pyrimidine), aminopurinol (4-aminopyrazolo(3,4-*d*) pyrimidine), 4-thiouridine and 3-deazauridine were from Aldrich, and pentamidine from Rhone–Poulenc Rorer. 9-Deazaguanine was generously provided by Howard Cottam of the University of California, San Diego. All other compounds such as unlabeled allopurinol, nucleosides, nucleobases and ionophores were from Sigma.

2.2.3 Media and growth chemicals

HOMEM medium and fetal bovine serum, were purchased from Invitrogen (Carlsbad, CA), Schneider's Drosophila medium and HMI-9 medium were from Gibco, and SDM79 medium was obtained from Life Technologies. β-NADH was obtained from Sigma-Aldrich. Alamar Blue™ was obtained from Trek Diagnostics (UK).

2.2.4 Enzymes, primers and reagents used for molecular technique

SuperScript™ II Reverse Transcriptase was purchased from Invitrogen. 1 kb DNA Ladder obtained from BioLabs or Invitrogen and *Taq* DNA Polymerase was purchased from Promega. All other enzymes for molecular techniques were obtained from either Invitrogen or Promega, and chemicals from Sigma. A set of primers used for PCR amplifications of AT-like sequences (AT-A F1, AT-A R1, AT-B F1, AT-B R1, AT-D F2, AT-D F3, AT-D R2, and AT-D R4) from *T. brucei* cDNA was designed by Lynsey Wallace. Primers used for PCR amplifications as controls (P2 F, P2 R, NBT1 R, NBT1 F, TbAT1 F1, and TbAT1 R1) were designed by Richard Burchmore, and Primers used as controls for genomic DNA (OL908, OL930, OL929, OL909) were kindly donated by

Gareth Westrop (see Appendix II for sequences and annealing temperatures for these primers).

2.3 Transport assays

2.3.1 Transport assays for *Leishmania major* promastigotes

Assays for transport of [^{3}H]adenine, [^{3}H]hypoxanthine and [^{3}H]allopurinol by *L. major* promastigotes was performed exactly as described for *Trypanosoma brucei* (De Koning, 2001; Wallace, *et al.*, 2002), using a rapid oil-stop protocol (Figure 2.1). Briefly, promastigotes were harvested and washed twice with the assay buffer pH 7.3 and re-suspended at ~10^8 cells/ml. Cells were then incubated with the radioligand in the presence or absence of inhibitor, ionophores were usually preincubated with the cells for 2-5 min. All ionophores were dissolved in ethanol and the uptake was measured against controls containing the same percentage of solvent, and spun through oil for 30 s at 13,000 rpm, after a predetermined time as appropriate for each experiment, but always within the linear phase of transport. All experiments were performed in triplicate at room temperature and non-mediated transport, in the presence of saturating permeant concentrations, was subtracted. Zero-uptake values were obtained in presence of 1 mM unlabeled permeant at 0°C. Radioactivity in the cell pellet was determined, after solubilisation in 2% SDS, by liquid scintillation counting.

2.3.2 Transport assays for *Leishmania mexicana* amastigotes

Transport of purine nucleosides/bases (adenosine, adenine, hypoxanthine and allopurinol) into extracellular *Leishmania mexicana* amastigotes- like form was performed using a modified oil-stop technique. Amastigotes forms of *L. mexicana* were harvested by centrifugation (2500g, 10 minutes) and washed twice in CBSS buffer pH 6.0. Cells were washed and resuspended in CBSS buffer at 32°C and used at

approximately 10^7 cells/ml. Transport was measured at 32°C, 100 µl of cell suspension was added to a microcentrefuge tube containing 200 µl oil [7:1 (by vol.) dibutyl-phthalate/mineral oil; d = 1.018 g/ml] and 100 µl [^{3}H]adenine (0.1 µM) or [^{3}H]hypoxanthine (0.03 µM) or [^{3}H]allopurinol (1 µM) and incubated for the indicated times. Incubations were terminated by adding 1ml ice-cold 4 mM of unlabeled permeant in CBSS buffer, and cells were separated from extracellular label by centrifugation (12000 × g, 30 s). The tubes were frozen in liquid nitrogen and the tips, containing the cell pellet, were cut off. The pellet was dissolved in 250 µl 2% SDS. After addition the scintillation fluid (Optiphase HiSafe III), radioactivity was determined in (1450 MicroBeta Trilax) scintillation counter. Zero uptake values were obtained by exposing the cells to a mixture of [^{3}H] permeant and 1 mM unlabeled permeant, both kept on ice until use. Non-mediated transport was determined by processing cell samples that had been mixed with [^{3}H] permeant in the presence of 4 mM permeant. Transport values were calculated after subtraction of the non-mediated values. In inhibition studies, test compounds were mixed with [^{3}H] permeant.

2.3.3 Transport assays for trypanosomes

The uptake of radiolabeled permeants by procyclic or bloodstream forms *T. b. brucei* (strain 427 or 927), was assessed exactly as described in section 2.3.1 for *Leishmania major* promastigotes, using a rapid oil-stop protocol. Bloodstream forms were harvested and washed at 4°C and cell suspensions were left for 20 minutes at room temperature before use.

2.3.4 Transport assays for *Toxoplasma gondii*

Transport of purine nucleosides and nucleobases (adenosine, adenine, hypoxanthine and inosine) into extracellular *T. gondii* tachyzoites was performed using a modified

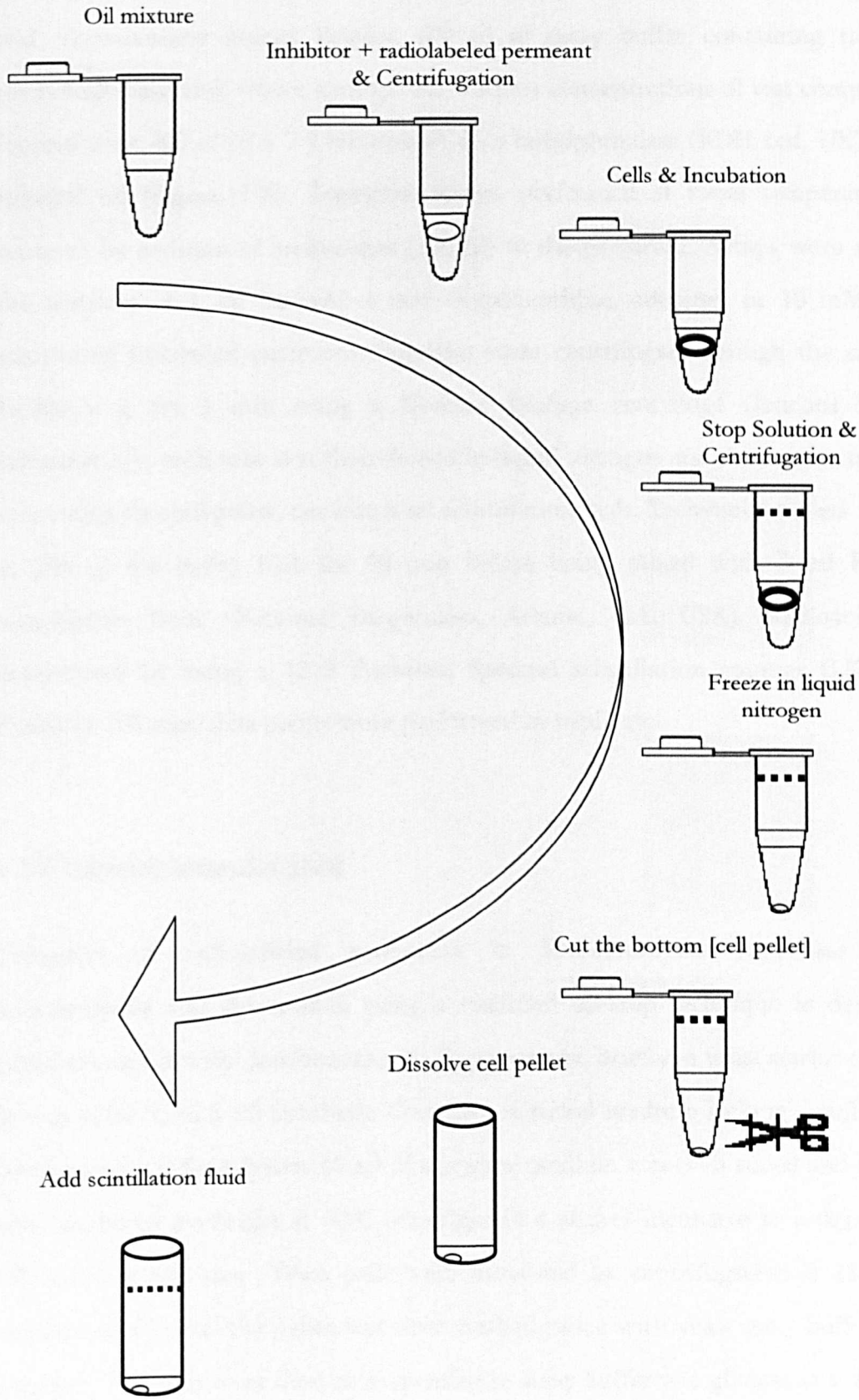


FIGURE 2.1 Schematic representation of the rapid oil-stop protocol used in our project with some modifications when required.

oil-stop technique previously described for transport measurements with *Leishmania* and *Trypanosoma brucei*. Briefly, 100 µl of assay buffer containing radiolabeled nucleoside/base and, where appropriate, various concentrations of test compound, was layered over 200 µl of a 7:1 mixture of di-*n*-butylphthalate (BDH Ltd, UK) and light mineral oil (Sigma, UK). Transport assays, performed at room temperature, were initiated by addition of tachyzoites (100 µl) to the permeant. Assays were stopped by the addition of 1 ml ice-cold 4 mM (hypoxanthine, adenine) or 10 mM (inosine, adenosine) unlabeled permeant. Parasites were centrifuged through the oil layer at 16,000 x g for 1 min using a Heraeus Biofuge centrifuge (Jencons PLS, UK). Subsequently, each tube was flash-frozen in liquid nitrogen and the bottom of the tube, containing the cell pellet, cut into 6 ml scintillation vials. Tachyzoite pellets were lysed in 250 µl 5% (w/v) TCA for 90 min before being mixed with 3 ml Ecoscint A scintillation fluid (National Diagnostics, Atlanta, GA, USA). Radioactivity was determined by using a 1219 RackBeta Spectral scintillation counter (LKB Wallac, Finland). All assay data points were performed in triplicate.

2.3.5 Transport assays for yeast

Transport of radiolabeled permeants in *Saccharomyces cerevisiae* MG887-1 transformants was determined using a modified oil-stop technique as described for transport assays with *Leishmania* and *Trypanosoma*. Briefly, a yeast starter culture was grown at 30 °C in 5 ml Synthetic Complete minimal medium lacking uracil (SC-URA) (see appendix I) for 6 hours; 45 ml of the same medium was then added and the culture was incubated overnight at 30°C overnight in a shaker-incubator to a density of 1–2 OD units at 600 nm. Yeast cells were harvested by centrifugation at 1800 g for 5 minutes at 4°C, and the pellet was then washed twice with yeast assay buffer (without glucose). The cells were then re-suspended in assay buffer w/o glucose at a density of ~ 10^8 /ml. The cell suspension was usually divided to three tubes which were kept on ice,

using each tube after incubation for 10 minutes at room temperature for one set of triplicates to minimize variation as much as possible.

2.4 Data analysis

Kinetic parameters were calculated using the FigP computer program (Elsevier Biosoft) and the Prism3 and Prism4 software packages (GraphPad Software, San Diego, CA), by using non-linear regression and variable slope. In most cases, Hill coefficients were found to be close to -1, consistent with monophasic competitive inhibition. In some cases, the Hill slope was <-1, apparently as a result of uptake of permeant by multiple transporters with different affinity for the inhibitor. In these cases, the data were fitted to equations for one-site competition and two-site competition and the results compared with an *F*-test.

All experiments were performed in triplicate and kinetic parameters are presented as means ± S.E of at least three independent experiments. K_i values were calculated from the equation: $K_i = IC_{50}/ [1 + (L/K_m)]$, in which L is the permeant concentration (Cheng and Prusoff, 1973). The Gibbs free energy of the transporter-ligand interaction was then calculated from the equation: $\Delta G° = - RT\ln(K_i)$, in which R is the gas constant and T the absolute temperature. It should be noted that these equations are only valid for competitive inhibition. In our experiments, transport of permeant refers to mediated transport minus radioactivity associated with the cells due to diffusion or binding. Therefore, the amount of radioactivity associated with the cell pellet in the presence of saturating levels of unlabeled permeant was subtracted from each data point. Errors given in tables and shown as bars in graphs are standard errors of the means (SEM). It should be noted that these equations are only valid for competitive inhibition, as described previously (De Koning and Jarvis. 1999; Wallace, *et al.*, 2002).

2.5 Drug sensitivity assay for *Leishmania mexicana* amastigotes

There are many methods for determining toxicity. Observation of the parasites for motility and viability after exposure to the potential drugs could be a simple method; however, it is time consuming. Alamar blue assay provides a simple assay to test compounds for anti-leishmanial activity in large-scale screening system. The advantages of using Alamar blue assay to measure cytotoxicity of compounds against the protozoa include: (I) the effect of drugs can be quantified without the need for cell counts, (II) non–radioactive, (III) simplicity, (IV) cheap, (V) non-toxic (Ahmed, *et al.*, 1994; Mikus and Steverding, 2000; Nakayama, *et al.*, 1997; Nociari, *et al.*, 1998; Raz, *et al.*, 1997; Habtemariam, 2003). The drug sensitivity assay with Alamar Blue was performed as described for *T. brucei* (Raz, *et al.*, 1997) and *Leishmania major* promastigotes (Mikus and Steverding, 2000) in 96-well plates with some modifications. Briefly, 100 µl of Schneider's Drosophila Medium (Gibco), used for culturing amastigotes, was added to each well in 96-well plate, leaving the first column empty. 200 µl of different test compounds was added, at double final concentration, to the first column. Doubling dilutions were performed, passing 100 µl from the first column to the next and repeating this across the plate but leaving the last well drug-free as negative control. Amastigotes form *Leishmania mexicana* were inoculated into each well at a final density of 1×10^6/ml culture medium. 20 µl of alamar blue reagent (TREK Diagnostic Systems, Ltd) was added after 48 h and fluorescence development determined after a further incubation time of 72 h. The plates were read using a LS55 Luminescence Spectrometer (Perkin Elmer) at wavelengths of 530 nm for excitation and 590 nm for emission.

2.6 Reverse Transcriptase (RT) – PCR

The reverse transcription polymerase chain reaction (RT-PCR) is a technique for enzymatically amplifying defined sequences of RNA (Rappolee, *et al.*, 1988). As RNA

can not serve as a template for PCR, the first step in this assay is the reverse transcription of the RNA template into complementary DNA (cDNA). Compared to the other commonly used techniques for quantifying mRNA levels, Northern blotting and in situ hybridisation (Parker and Barnes, 1999), RNase protection assay (Hod, 1992; Saccomanno, *et al.*, 1992), the RT-PCR can be used to quantify mRNA levels from much smaller samples. In fact, this technique is the most sensitive, as it permits the analysis of different samples from as little as one cell in the same experiment, and the most flexible of the quantification methods (Wang and Brown, 1999).

2.6.1 RNA extraction from different strains and life cycle stages of *T. brucei*

Parasites from different life cycle stages of *T. brucei* (Procyclic from a 2 day old culture of *T. brucei* strain 427, long-slender bloodstream forms of *T. brucei* strain 427 in HMI-9 medium, and long-slender or short-stumpy bloodstream forms of *T. brucei* strain 927 isolated from a DE52 column) were counted using a Neubauer haemocytometer. 1 ml containing 1×10^7 cells was transferred to sterile RNase free eppendorf tubes. Cells were harvested by centrifugation at 1000 g for 3 minutes at 4°C. 1 ml of Tri reagent (Sigma) was added and mixed gently by pipetting. Cells were left to lyse completely at room temperature for 5 minutes. 200 µl of RNase free chloroform (Sigma) was added and the suspension was vortexed for 15 seconds, the tube was then centrifuged at 13,000 g for 15 minutes at 4°C and the upper aqueous layer (containing RNA) was transferred to fresh eppendorf tube. An equal volume of isopropanol was added and mixed, fallowed by incubation at room temperature for 10 minutes. The tube was centrifuged at 13,000g for 15 minutes at 4°C to pellet the RNA, which was then washed with 500 µl of 75% ethanol and centrifuged at 7500g for 5 minutes at 4°C. Ethanol was removed and allowed to air dry at room temperature for 10 minutes. The pellet was resuspended in 30 µl of DEPC (diethylpyrocarbonate)-treated dH_2O and incubated at 55°C for 10 minutes. Concentrations of RNA were measured using either Nanospec or Agilent 2100

Bioanalyzer. RNA samples were stored at – 70°C for experimental use. Note: all materials from reagents to equipment used were exclusive for RNA work to avoid any contaminations.

2.6.2 First-Strand cDNA synthesis

First strand cDNA synthesis was done using a SuperScript™ II Reverse Transcriptase (RT) (Invitrogen), which is a DNA polymerase that synthesizes a complementary DNA strand from single-stranded RNA, DNA, or an RNA:DNA hybrid. This enzyme is genetically engineered by the introduction of point mutations to have reduced RNase H activity, while maintaining full polymerase activity. This structural modification eliminates degradation of RNA molecules during first-strand cDNA synthesis. This enzyme provides greater first-strand cDNA yields, more full-length cDNA and activity up to 50°C. Using sterile RNase free eppendorf tubes, 1 µl of oligo $(dT)_{12\text{-}18}$, 1 ng to 5 µg of total RNA (depending on the concentration of RNA), 1 µl of 10 mM dNTP Mix (10 mM each dATP, dGTP, dCTP and dTTP), and sterile distilled water to 12 µl were added. The mixture was heated to 65 °C for 5 minutes and quick chilled on ice and the contents of the tube was collected by brief centrifugation. 4 µl of 5× First-Strand Buffer, 2 µl of 0.1 M DTT, and 1 µl of RNaseOUT (Invitrogen) were then added, and the contents of the tube were mixed gently and incubated at 42 °C for 2 minutes. 1 µl of SuperScript II RT was added and mixed by pipetting gently up and down and then the tube was incubated for 50 minutes at 42 °C. Finally, the reaction was inactivated by heating at 70 °C for 15 minutes. Control tubes were treated exactly in the same way but without adding the SuperScript II RT enzyme. Samples of cDNA and the controls were stored at -20 °C to use as a template for amplification in PCR.

2.6.3 Genomic DNA extraction from different life cycle stages of *T. brucei*

Procyclic or bloodstream forms of *T. brucei* were centrifuged at 3500 rpm for 15 min at 4 °C. Cell pellets were then re-suspended in 450 µl DNA extraction buffer (see Appendix I). 50 µl 10% SDS was added followed by 25 µl (10 mg/ml) proteinase K. Tubes were then incubated for 2 hrs at 37 °C while mixing gently on a rocking platform. 500 µl of liquid phenol was added and vortexed thoroughly (solution becomes milky in appearance), followed by centrifugation for 5 minutes at 13,000 g. The top layer was transferred into a fresh tube and an equal volume of phenol-chloroform (50:50) was added, usually about 500 µl. The tube was vortexed thoroughly and centrifuged for 5 minutes at 13,000 g, and the upper aqueous layer was transferred to a fresh tube and equal volume (500 µl) chloroform was added. Tube was vortexed thoroughly and centrifuged for 5 minutes at 13000 g, and the top layer was then transferred to fresh tube (400 µl). 40 µl of 3 M Na-acetate plus 1 ml of 100% ice cold ethanol were added, and the tube was then left at -20 °C for about 30 minutes. The tube was centrifuged for 15 minutes at 13,000 g to pellet DNA, and then washed with 70% ethanol. The pellet was dried in a 37 °C incubator for 10-15 minutes and re-suspended in 50 µl of dH_2O or TE buffer and then stored at -20 °C.

2.6.4 Polymerase Chain Reaction (PCR) for DNA

The PCR was performed in a reaction mixture of 20 µl containing 2 µl of 10× *Taq* buffer, 1 µl of 25mM $MgCl_2$, 0.4 µl of 10 mM dNTP mix (200 µM of each of dATP, dCTP, dTTP, dGTP), 1 µl of each primer (2.5 µM), 0.4 *Taq* DNA polymerase (5U/µl). 1 µl of either cDNA, the negative control (from first-strand reaction), or genomic DNA (positive control) was added, followed by autoclaved distilled water (dH2O) to a final 20 µl volume. A negative control was performed in all of our PCR reactions which was exactly the same reaction mixture tube but without cDNA, or using the sample from

the negative control (from first-strand reaction). PCRs were performed using the PTC-200 Peltier Thermal Cycler (MJ Research). Two different programmes were used for the annealing temperature and the cycle conditions, depending on the primers used. PCR conditions consisted in a hot-start denaturation for 2 minutes 94°C, followed by 30 cycles of 94°C for 30 seconds, annealing temperature for 30 seconds (temperature dependent on the particular primers), and extension at 72°C for 2 minutes, followed by a final extension step at 72°C for 10 minutes.

2.6.5 Agarose Gel Electrophoresis of DNA

Agarose powder was mixed with TBE (Tris-borate-EDTA) electrophoresis buffer to the needed concentration (usually 0.8% or 1.2%), then heated in a microwave oven until completely melted. Ethidium bromide (final concentration of 0.3 μg/ml) was added to the gel at this point to facilitate visualization of DNA after electrophoresis. After cooling the solution to about 60°C, it was poured into a casting tray containing a sample comb and allowed to solidify at room temperature. Samples containing DNA or controls mixed with loading buffer (5-7 μl) were then pipeted into the sample wells (usually 10 μl per well). The voltage used to run the gels was normally 60 V, and gel visualization was by using UV transilluminator (UVP Products). 1 kb DNA Ladder (BioLabs or Invitrogen) was used for determining the size of double-stranded DNA.

Chapter Three

CHARACTERISATION OF A NOVEL NUCLEOBASE TRANSPORTER IN *LEISHMANIA MAJOR* PROMASTIGOTES

3.1 Summary

Despite the severe side-effects and emerging resistance, pentavalent antimony drugs such as Pentostam (sodium stibogluconate) or Glucantime (N-methylglucamine antimoniate) are still widely used to treat human leishmaniasis (Croft, 2001; Sundar, 2001b; Croft, *et al.*, 2005; Soto, *et al.*, 2005). Indeed, the hypoxanthine analogue allopurinol is one of only a hand-full of drugs clinically used against various forms of leishmaniasis, which, either alone or in combination with other drugs, has proved effective against cutaneous (Martinez and Marr, 1992; Baum and Berens, 1994; Becker, *et al.*, 1999), ocular (Abrishami, *et al.*, 2002) or visceral leishmaniasis (Llorente, *et al.*, 2000; Das, *et al.*, 2001; Momeni, *et al.*, 2002; Pasa, *et al.*, 2005). Several of these reports describe combinations of allopurinol with low doses of other antileishmanials that are more effective than the usual dosage of the other drug alone and the reduced dosage of pentamidine or antimony reduces or eliminates harmful side effects. Combination therapy also delays onset of drug resistance.

Purine nucleobase transporters play central roles in the biochemistry of parasitic protozoa such as *Leishmania*, because these parasites cannot synthesize purines *de novo* and are absolutely reliant upon purine salvage from their hosts (Carter, *et al.*, 2003). In contrast, almost all mammalian cells can synthesis purines *de novo* and this difference between parasitic and mammalian cells results in purine salvage being a potential target for chemotherapy (Craig and Eakin, 1997). Furthermore, nucleobase transporters are important to the pharmacology of these significant human pathogens, because they mediate the uptake of purine analogues, as well as some non-purine drugs, that are selectively cytotoxic to the parasites. In the last few years major progress has been made in the biomedical and molecular characterisation of the purine nucleobase transporters. However, little is known about the way their expression or activity is regulated and a thorough understanding of these processes is vital for the effective exploitation of transporters to deliver chemotherapeutic agents.

The metabolism of allopurinol in *Leishmania* species to the active metabolite, 4-aminopyrazolo(3,4-*d*)pyrimidine ribonucleoside triphosphate, has been described (Nelson, *et al.*, 1979a; Marr and Berens, 1983). To date, however, no study has addressed allopurinol uptake by this parasite, even though the issue is important in understanding the selectivity of the drug as well as the potential for the development of resistance. In the related kinetoplast, *Trypanosoma brucei*, allopurinol is taken up through high affinity purine nucleobase transporters (De Koning and Jarvis, 1997a; De Koning and Jarvis, 1997b; Natto, *et al.*, 2005). We have therefore conducted a comprehensive study of purine nucleobase uptake in *Leishmania major* promastigotes. We have identified a single transporter, designated LmajNBT1, with high affinity for all physiological purine bases and moderately high affinity for allopurinol. Studies with [^{3}H]allopurinol confirmed that this transporter is its sole route of entry into promastigotes.

In this chapter, the uptake of [^{3}H]adenine, [^{3}H]hypoxanthine, and [^{3}H]allopurinol were investigated. These compounds were all taken up by a single high-affinity transporter, designated LmajNBT1, with K_m values of 4.6 ± 0.9, 0.71 ± 0.07, and 54 ± 3 μM, respectively. Guanine and xanthine fully inhibited [^{3}H]adenine transport, with Ki values of 2.8 ± 0.7 and 23 ± 8 μM.

A model for the interactions of LmajNBT1 with its substrates was constructed using the techniques developed to study *Trypanosoma brucei* and human purine transporters (De Koning and Jarvis, 1999; Wallace, *et al.*, 2002) to allow wider predictions about the potential of drug uptake by LmajNBT1. The resulting model predicts that this transporter could mediate the uptake of an extensive range of nucleobase and guanosine analogues. In addition, it was shown that the architecture of the *L. major* and *T. brucei* nucleobase transporters are similar enough to bind purine bases in almost identical fashion, though the nucleobase transporter expressed in human erythrocytes and other cell types binds the same substrates in an entirely different way. This implies

that many nucleobase analogues could be selectively internalised by both protozoan pathogens but remain excluded from many host cells. Though functional relationships such as highlighted in this study do not necessarily reflect evolutionary relationships, they have more pharmacological relevance than gene sequence alignments. This work has, in part, been previously published as Al Salabi et al. (2003) Mol. Pharmacol. 63:814-820 (see Appendix III).

3.2 Results

3.2.1 Adenine transport by *Leishmania major* promastigotes

At the start of this project, the number of nucleobase transporters in *L. major* and their kinetic properties were unknown. Thus, initial studies were performed to investigate nucleobase transport in *L. major* and to compare the transport properties with those reported for *T. brucei*. Transport of 1 μM [^{3}H]adenine by mid-log phase *L. major* promastigotes (Figure 3.1) at 22 °C was linear for at least 25 s (Figure 3.2, *inset*), with a rate of 1.1 ± 0.1 pmol(10^7 cells)$^{-1}$s^{-1}, whereas in the presence of 1 mM unlabeled adenine no uptake of [^{3}H]adenine was detectable over 120 s (Figure. 3.2), indicating that transport was saturable. [^{3}H]Adenine uptake, measured over 10 s, followed Michaelis-Menten kinetics (Figure 3.3, *inset*) and displayed an apparent K_m and V_{max} values of 4.6 ± 0.9 μM and 3.2 ± 0.3 pmol(10^7 cells)$^{-1}$s^{-1}, respectively, (n = 3). This experiment was performed at 0.1 μM of label and assumed to be linear over the short incubation period (10 s). Considering the much longer linear uptake phase at 1 μM of label, it is felt this is a reasonable assumption.

Transport of [^{3}H]adenine was inhibited by a range of purine nucleosides and nucleobases (Table 3.1), but not by the pyrimidines uracil, cytosine, thymine and thymidine at concentrations up to 1 mM ($P > 0.05$; Unpaired Student's T-test). Nor was [^{3}H]adenine transport significantly inhibited by 25 μM dilazep or dipyridamole (Figure

3.4). The adenine transporter generally displayed far higher affinity for nucleobases than for their corresponding nucleosides, as illustrated in Figure 3.3 for adenine and adenosine, which displayed a K_i value of > 5 mM (n = 3). Allopurinol was a moderately effective inhibitor of [^{3}H]adenine transport (Figure 3.3) with a K_i of 56 ± 2 μM (n = 3). All inhibition profiles displayed Hill coefficients near –1 and maximum inhibition was invariably equal to the level of inhibition of the control (1 mM unlabelled adenine). These observations are consistent with a single transport activity for [^{3}H]adenine.

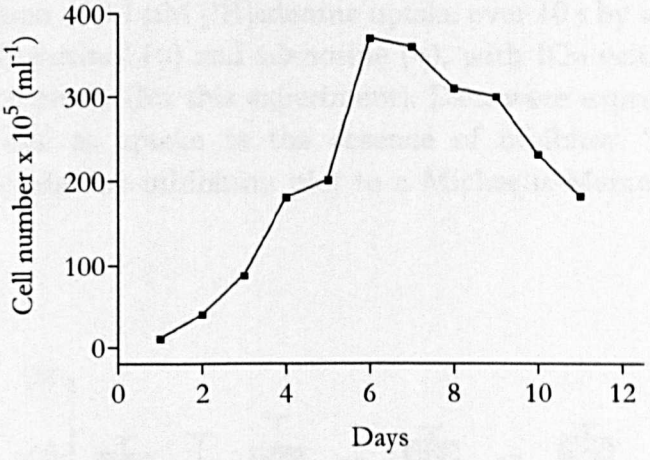


FIGURE 3.1 Growth curve of *L. major* promastigotes in Homem medium + 10% heat-inactivated fetal calf serum at 25 °C.

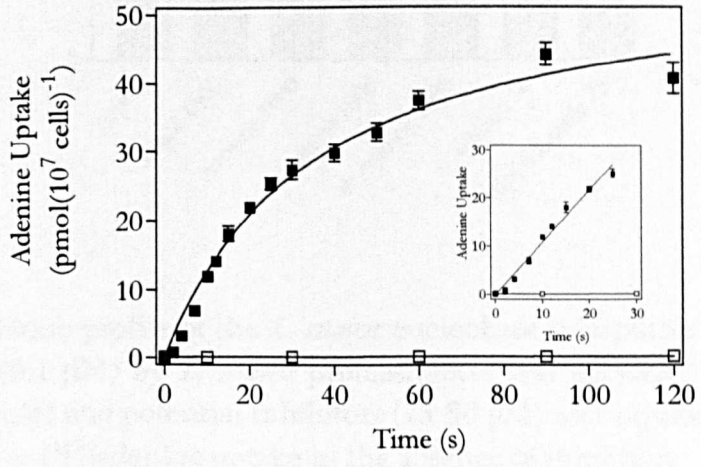


FIGURE 3.2 Timecourse of 1 μM [^{3}H]adenine uptake by *L. major* promastigotes in the presence (□) or absence (■) of 1 mM unlabelled adenine. The inset shows the linear phase of this curve (25 s). The line was calculated by linear regression (r^2 = 0.97).

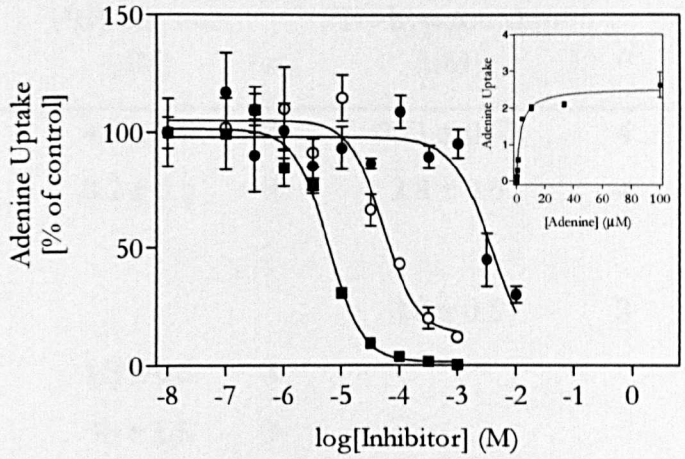


FIGURE 3.3 Inhibition of 0.1 μM [^{3}H]adenine uptake over 10 s by various concentrations of adenine (■), allopurinol (○) and adenosine (●), with IC_{50} values of 5.9 μM, 52 μM and 3.9 mM, respectively (for this experiment). Data were expressed as percentage of the control, defined as uptake in the absence of inhibitor. The inset shows the conversion of the adenine inhibition plot to a Michaelis-Menten curve (in pmol(10^7 cells)$^{-1}$s^{-1}).

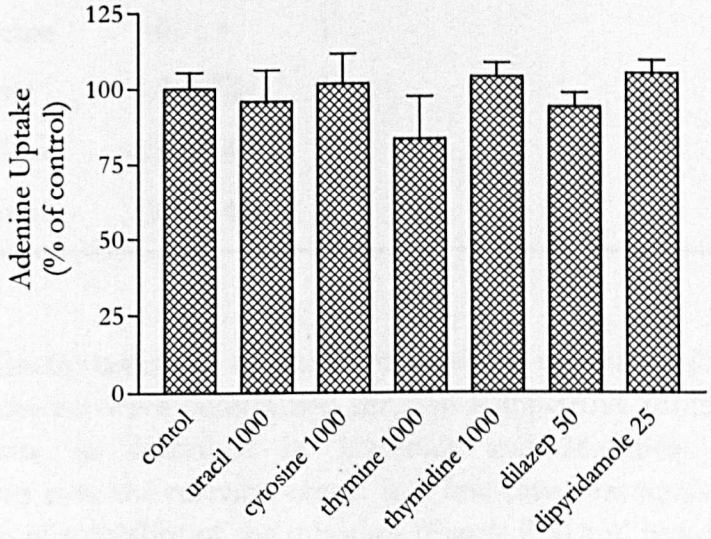


FIGURE 3.4 Inhibition profile of the *L. major* nucleobase transporter LmajNBT1. Uptake of [^{3}H]adenine (0.1 μM) by *L. major* promastigotes was measured in the presence of pyrimidines (1 mM) and potential inhibitors (25-50 μM) and expressed as percentage of control, defined as [^{3}H]adenine uptake in the absence of inhibitor.

	[^{3}H]-adenine (μM)	*n*	[^{3}H]-hypoxanthine (μM)	*n*	[^{3}H]-allopurinol (μM)	*n*
K_m	4.6 ± 0.9	3	0.71 ± 0.07	4	54 ± 2.9	3
V_{max} [a]	3.2 ± 0.3	3	2.8 ± 0.6	4	0.24 ± 0.06	3
K_i values						
adenine			3.0 ± 0.5	3		
hypoxanthine	1.3 ± 0.3	3			0.30 ± 0.09	3
allopurinol	56 ± 1.5	3				
guanine	2.8 ± 0.7	4				
xanthine	23 ± 8	3				
adenosine	5150 ± 550	3				
inosine	125 ± 15	3				
guanosine	68 ± 17	4				
purine	6.7 ± 0.4	3				
1-deazapurine	26 ± 4.1	3				
3-deazaguanine	48 ± 5	3				
6-thioguanine	6.2 ± 0.8	3				
7-deazaguanine	426 ± 140	3				
9-deazaguanine	204 ± 4	3				

TABLE 3.1 Kinetic constants of purine nucleobase uptake in *L. major* promastigotes. Kinetic parameters were determined through competitive inhibition of the indicated [^{3}H]-permeants, as described in Materials and Methods, with 8-11 inhibitor concentrations over the relevant range. In a few cases, extrapolation was required due to limitations of solubility of the inhibitor (Figure 3.3) and based on the assumption of a Hill slope of –1 and eventual 100% inhibition. Extrapolation was not attempted when inhibition at the highest inhibitor concentration was <50%. Permeant concentration was 0.1 μM (adenine and hypoxanthine) or 0.5 μM (allopurinol). [a] expressed as pmol(10^7 cells)$^{-1}$s^{-1}.

3.2.2 A single transporter is responsible for uptake of purine nucleobases

The inhibition of adenine transport by other purine bases does not establish whether these bases are in fact transported across the plasma membrane by this adenine transporter, nor whether additional transporters for purine nucleobases are expressed in *L. major* promastigotes. To further investigate these issues, additional transport studies were performed using [^{3}H]hypoxanthine and [^{3}H]allopurinol. [^{3}H]Hypoxanthine uptake (0.1 μM) was linear for at least 15 sec (Figure 3.5, *inset*), with a rate of 0.22 ± 0.02 pmol(10^7 cells)$^{-1}$s^{-1}, whereas in the presence of 1 mM unlabelled hypoxanthine no uptake of [^{3}H]hypoxanthine was detectable over 120 s (Figure 3.5), indicating that transport was saturable. The apparent K_m value was 0.71 ± 0.07 μM and [^{3}H]hypoxanthine transport was inhibited by adenine with a K_i value of 3.0 ± 0.5 μM (Figure 3.6). The K_m is therefore almost identical to the K_i value for hypoxanthine inhibition of adenine transport, and the K_i value for adenine inhibition of [^{3}H]hypoxanthine transport is equal to the K_m for adenine uptake (Table 3.1).

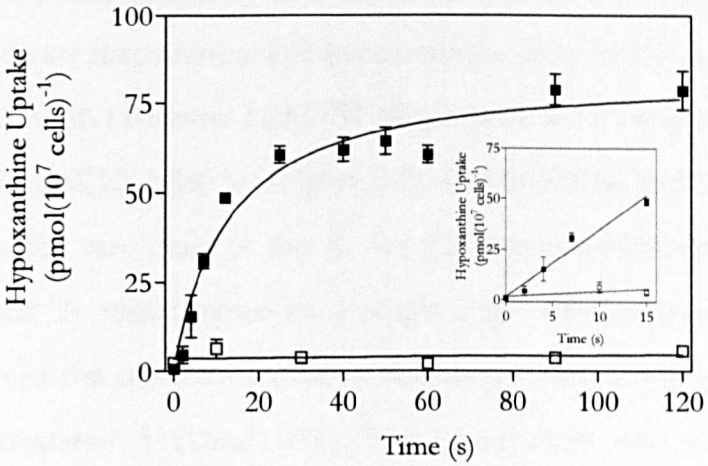


FIGURE 3.5 Time course of 0.1 μM [^{3}H]hypoxanthine uptake in the presence of unlabelled hypoxanthine (□) over 15 s was linear (r^2 = 0.92) and not significantly different from zero (P > 0.05) or in the absence (■) of 1 mM unlabelled hypoxanthine. The inset shows that the uptake was linear over 15 s (r^2 = 0.96) as calculated by linear regression

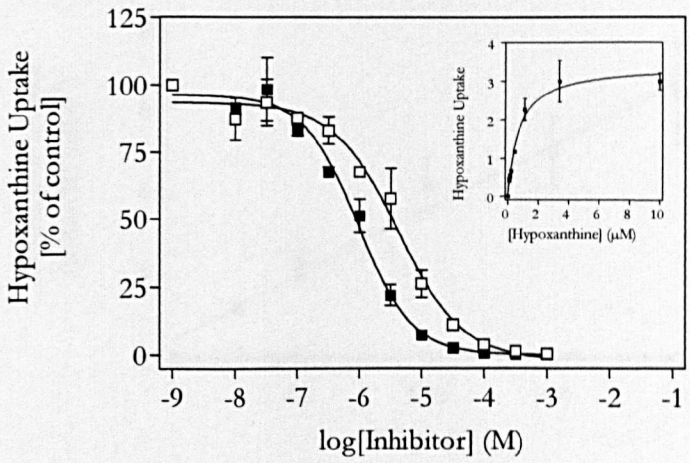


FIGURE 3.6 Transport of 0.1 μM [^{3}H]hypoxanthine over 10 s was inhibited by indicated concentrations of unlabelled hypoxanthine (■) or adenine (□), with IC_{50} values of 0.91 μM and 4.1 μM, respectively. The inset depicts the conversion of the hypoxanthine inhibition data to a Michaelis-Menten plot of total hypoxanthine uptake, with a K_m value of 0.69 μM and a V_{max} of 3.4 pmol(10^7 cells)$^{-1}$s^{-1} for this experiment.

In addition, purine nucleosides inhibited transport of 0.1 μM [^{3}H]hypoxanthine in a manner similar to [^{3}H]adenine: 1 mM adenosine, 1 mM inosine and 250 μM guanosine inhibited hypoxanthine uptake by 32 ± 13, 94 ± 1% and 87 ± 1%, respectively (n = 3). These results indicate that adenine and hypoxanthine most likely compete for uptake at a single transport unit. Likewise, 1 μM [^{3}H]allopurinol, which was taken up with a rate of 0.026 ± 0.002 pmol(10^7 cells)$^{-1}$s^{-1} (Figure 3.7), was inhibited by hypoxanthine (Figure 3.8) with a K_i value very close to the K_m for [^{3}H]hypoxanthine uptake (Table 3.1). It thus appears that *L. major* expresses a single high affinity transporter for purine nucleobases. Given the substrate profile of this transporter, it was designated *L. major* Nucleobase Transporter 1 (LmajNBT1). This transporter was not sensitive to the transporter inhibitors dilazep and dipyridamole, which inhibited 0.1 μM [^{3}H]hypoxanthine transport by just 20 ± 4% and 26 ± 4%, respectively, at 50 μM (n =3).

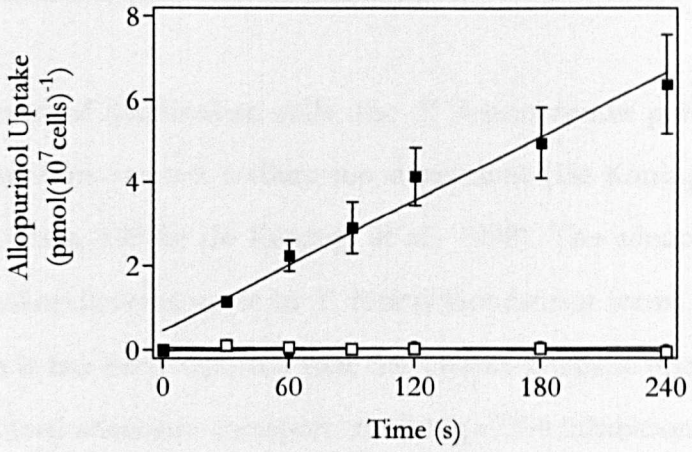


FIGURE 3.7 Timecourse over 240 s, at a final [^{3}H]allopurinol concentration of 1 μM. 10^7 promastigotes were incubated for up to 240 s without inhibitor (■), in the presence of 250 μM hypoxanthine (□) or 1 mM allopurinol (●). The rate of uptake was calculated by linear regression (r^2 = 0.96). Allopurinol transport was not significantly different from zero in the presence of hypoxanthine or unlabelled allopurinol. The intercept of 1 μM allopurinol was 0.82 ± 0.29 pmol(10^7 cells)$^{-1}$ and not significantly different from zero (P< 0.05).

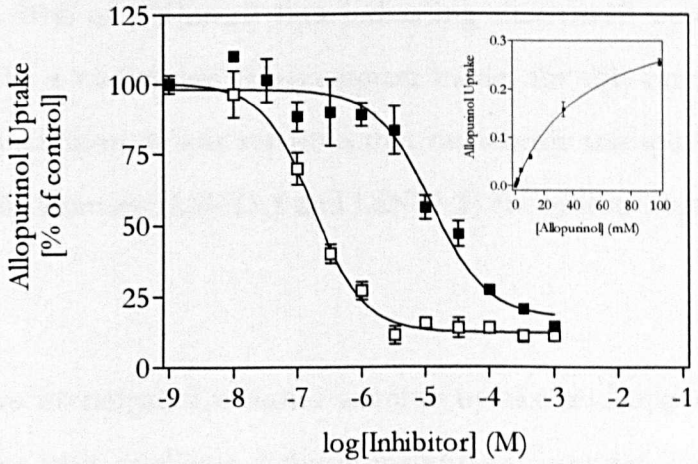


FIGURE 3.8 Uptake of 0.5 μM [^{3}H]allopurinol over 120 s was inhibited by up to 1 mM unlabelled allopurinol (■) or hypoxanthine (□). The allopurinol inhibition plot was converted to a Michaelis-Menten plot (*inset*), with a K_m value of 50 ± 3.6 μM and a V_{max} of 0.39 ± 0.01 pmol(10^7 cells)$^{-1}$s^{-1} for this experiment.

3.2.3 Ionic requirements for adenine transporter

In contrast to most of mammalian cells, the *T. brucei brucei* purine nucleobase and nucleoside transporters are not sodium ion-dependent (De Koning and Jarvis, 1997a; De Koning and Jarvis, 1997b; De Koning, *et al.*, 1998). The adenosine transporter P1 was the first nucleoside transporter in *T. brucei* bloodsteam forms that appeared to be proton-linked as it has been reported that the proton-gradient uncoupler CCCP dose-dependently reduced adenosine transport, reaching >75% inhibition at 20 μM. The 50% effective concentration of CCCP was 2.5 ± 0.4 μM. In addition, similar reductions in adenosine transport were seen with the ionophore nigericin and the Na^+/K^+ exchanger gramicidin. In contrast, neither monensin, a Na^+ ionophore, nor replacement of Na^+ with NMG^+ had any effect on adenosine transport rates (De Koning, *et al.*, 1998). It has also been demonstrated that the hypoxanthine transporters (H1 and H2) in *T. b. brucei* procyclic and bloodstream forms are nucleobase/proton symporter as they are linked to a protonmotive force (De Koning and Jarvis, 1997a; De Koning and Jarvis, 1997b). Furthermore, the proton ionophore carbonyl cyanide chlorophenylhydrazone (CCCP) inhibited up to 70% of [^{3}H]uracil flux indicating that uracil uptake in *T. b. brucei* procyclics may be a nucleobase-H+-symporter model for this carrier (De Koning and Jarvis, 1998). In addition, it was reported that nucleoside transporter family members from *Leishmania donovani* (LdNT1.1 and LdNT1.2) are proton-dependent (Stein, *et al.*, 2003).

In this study, we investigated whether adenine uptake in *L.major* promastigotes was dependent on the ionic gradients defining membrane potential. The proton uncoupler CCCP (carbonyl cyanide chlorophenylhydrazol), the membrane depolarization agent gramicidin and the K^+/H^+ -exchanger nigericin were tested. All inhibited adenine uptake in a dose-dependent manner (Figure 3.9).

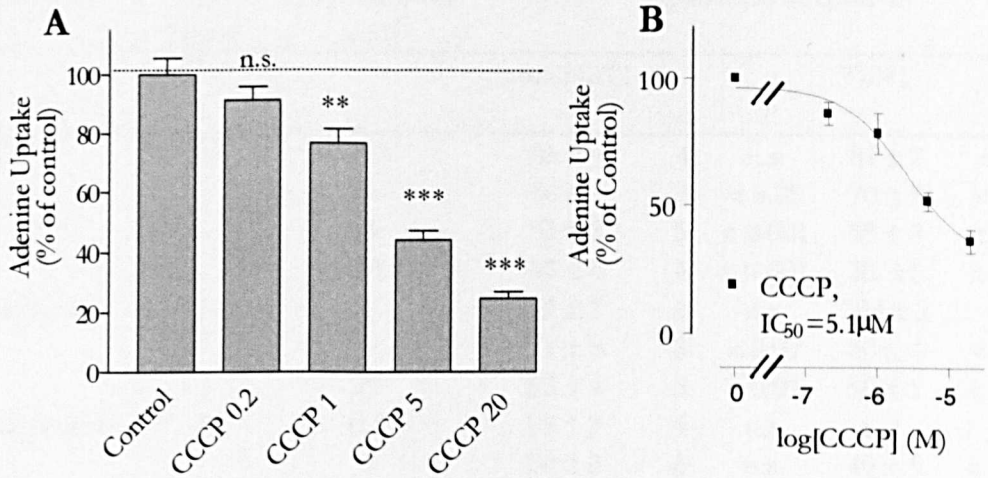


FIGURE 3.9 Effects of different concentrations (0.2-20 μM) of the proton uncoupler CCCP (carbonyl cyanide chlorophenylhydrazol) on LmajNBT1-mediated 0.1 μM [^{3}H]adenine transport. A. using unpaired student's *T*-test for statistical significance analysis; **, $P < 0.02$, ***, $P < 0.001$, and n.s., not significant. B. IC_{50} plot for effect of CCCP using the values listed in Table 3.2.

The result was not unexpected as earlier studies show that solute transport in protozoan is usually dependent on the proton gradient rather than sodium gradient. Certainly the results with CCCP were very similar to the earlier results with various proton symporters of *T. brucei*: CCCP IC_{50} = 5.1 μM for LmajNBT1 (Figure 3.9*B*), CCCP IC_{50} = 3.8 μM for TbU1 (De Koning and Jarvis, 1998), CCCP IC_{50} =2.5 μM for TbH1 (De Koning and Jarvis, 1997a).

Nevertheless, LmajNBT1-mediated adenine transport was less sensitive to gramicidin than the corresponding transport activity in *T. b. brucei* procyclics (Table 3.2). The findings reported here are broadly supportive of proton/nucleobase symport in *L. major* promastigotes but additional studies will be required for the necessary degree of certainty for such a conclusion.

Compound	P.I. [min]	Concentration [μM]	Uptake [% of control]				
			LmajNBT1			TbH1	
				n	*P*		*P*
CCCP	2	0.2	86 ± 4	4	n.s.	81 ± 2	< 0.01
		1	70 ± 5	3	< 0.05	70 ± 5	< 0.01
		5	51 ± 3	5	< 0.001	55 ± 3	< 0.001
		20	35 ± 4	4	< 0.001	31 ±1	< 0.001
Nigericin	3	1	92 ± 1	3	n.s.	104 ± 2	n.s.
		5	71 ± 3	3	< 0.02	80 ± 4	< 0.02
		20	63 ± 4	3	< 0.01	50 ± 1	< 0.001
Gramicidin	3	0.2	98 ± 3	4	n.s.	n.d.	
		1	88 ± 3	6	n.s.	40 ± 5	< 0.001
		5	67 ± 4	7	< 0.001	n.d	
		10	n.d			33 ± 1	< 0.001
		20	57 ± 5	6	< 0.05	n.d	
		25	n.d			32 ± 2	
		50	50 ± 16	3	< 0.05	n.d	

TABLE 3.2 Effects of agents affecting transmembrane ionic gradient on adenine transport. Promastigotes of *L. major* cells were preincubated (P.I.) under the indicated conditions, and the influx of 0.1 μM adenine for 10 s was subsequently determined as described in (chapter two). Results are expressed as means ± S.E. of 3-7 independent experiments, each preformed with 6 replicates per concentration group. Data for the *T. b. brucei* H1 transporter was obtained from (de Koning and Jarvis, 1997a). Statistical significance was measured against uptake in presence or absence of the inhibitors. n.s., not significant. n.d. not determined.

3.2.4 Structure-activity relationships of LmajNBT1

The fact that LmajNBT1 displays broad selectivity for purine nucleobases and mediates the uptake of allopurinol suggests that it may also transport other potential purine antimetabolites. The antineoplastic drug 6-thioguanine, for instance, displayed a K_i value of 6.2 ± 0.8 μM for LmajNBT1, and is probably efficiently taken up through this transporter. It has been demonstrated that models for the interactions between a transporter binding pocket and its permeant can be constructed through the study of competitive inhibition by structural analogues, and that such models have predictive value with respect to substrate recognition (De Koning and Jarvis, 1999; Wallace, *et al.*,

2002; De Koning, *et al.*, 2003; De Koning, *et al.*, 2005). A model for permeant recognition by LmajNBT1 is displayed in Figure 3.10*A*. The proposed hydrogen bond between LmajNBT1 and N3 follows most directly from the observation that N3 is essential for high affinity binding: 3-deazaguanine displays >10-fold lower affinity than guanine (Table 3.1). Conversion of the K_i values to Gibbs free energy as described in data analysis in chapter two, yields an energy difference $\delta(\Delta G^0)$ of 7.0 kJ/mol (Table 3.3), the apparent energy of the H-bond lost in 3-deazaguanine. Similar comparisons of guanine with 7-deazaguanine and 9-deazaguanine reveal H-bonds of 12.5 and 10.6 kJ/mol with N7 and N(9)H, respectively. The $\delta(\Delta G^0)$ of 3.3 kJ/mol between purine and 1-deazapurine is small but highly significant ($P < 0.02$) and shows that a weak interaction is formed between N1 of the purine ring and an H-bond donor in the LmajNBT1 binding site. In contrast, no significant difference in affinity was observed between purine and adenine, or between guanine and 6-thioguanine, showing that the functional groups at position 6 do not contribute to binding. The $\delta(\Delta G^0)$ of 5.6 kJ/mol between purine and hypoxanthine must therefore be the result of binding through N(1)H. Since the weak bond with the unprotonated N1 of purine (3.3 kJ/mol) is lost in hypoxanthine, the Gibbs free energy of the H-bond with N(1)H must be 8.9 kJ/mol.

Corroborating evidence for this model is obtained from the K_i values of other compounds listed in Table 3.1. The structure of allopurinol differs from hypoxanthine in that N7 has shifted to position 8, and the $\delta(\Delta G^0)$ of 10.8 kJ/mol is virtually identical to the estimated bond energy for N7 (Figure 3.10*A*). The low affinity for nucleosides ($\delta(\Delta G^0)$ >10 kJ/mol) is consistent with the proposed H-bond to N(9)H. The $\delta(\Delta G^0)$ of xanthine versus hypoxanthine (8.6 kJ/mol) is identical to the estimated bond energy for N3, which is protonated in xanthine. Figure 3.10 also explains the total lack of recognition of pyrimidine nucleobases. Finally, the sum of the individual bond energies is within 10% of the observed ΔG^0 for adenine and hypoxanthine as calculated from their K_m values. For hypoxanthine, $\Sigma(\Delta G^0)$ = -39.0 kJ/mol, while the ΔG^0_{obs} = -35.1 kJ/mol and for adenine these values are -33.4 and –30.5 kJ/mol, respectively.

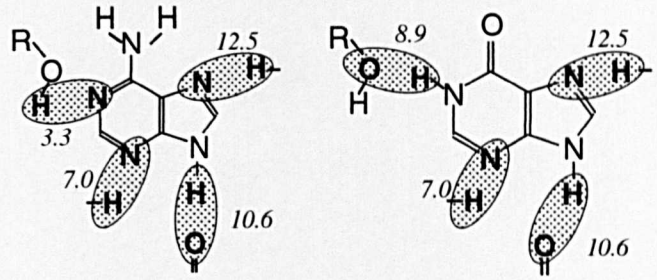
	LmajNBT1		H2		
Compound	(ΔG^0)	$\delta(\Delta G^0)$	(ΔG^0)	$\delta(\Delta G^0)$	Control
Hypoxanthine	-35.1		-39.5		
Guanine	-31.7	3.4	-36.8	2.7	Hypoxanthine
Adenine	-30.5	4.6	-31.4	8.1	Hypoxanthine
Xanthine	-26.5	8.6	-28.9	10.6	Hypoxanthine
Allopurinol	-24.3	10.8	-30.8	8.7	Hypoxanthine
Guanosine	-23.8	7.9	-28.3	8.5	Guanine
Inosine	-22.3	12.8	-23.7	15.8	Hypoxanthine
Adenosine	-13.1	17.4	-17.4	12.9	Adenine
Purine	-29.5	0.9	-27.0	4.4	Adenine
1-Deazapurine	-26.2	3.3	-26.6	0.4	Purine
3-Deazaguanine	-24.7	7.0	-28.5	8.3	Guanine
6-Thioguanine	-29.7	2.0	-36.3	0.5	Guanine
7-Deazaguanine	-19.2	12.5	-24.3	12.5	Guanine
9-Deazaguanine	-21.1	10.6	-29.1	7.7	Guanine

TABLE 3.3 Gibbs free energies (kJ/mol) of substrate interacting with LmajNBT1 or the *Trypanosoma brucei brucei* H2 transporter. Gibbs free energy of substrate-transporter interactions was calculated from the K_m and K_i values listed in Table 3.1, using the Nernst equation as described in Materials and Methods. The difference with a control compound, either hypoxanthine as the highest affinity compound, the corresponding physiological nucleobase (in the case of chemical analogues) or (in the case of nucleosides) the corresponding nucleobase yielded the $\delta(\Delta G^0)$, the loss in binding energy relative to the control compound. The values for the *T. b. brucei* H2 transporter were transcribed from (Wallace, *et al.*, 2002).

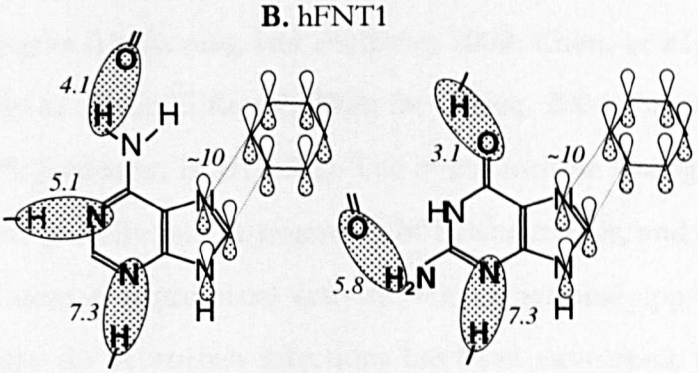
The proposed model for substrate binding by LmajNBT1 differs greatly from the one recently reported for the human facilitative nucleobase transporter hFNT1 (Figure 3.10*B*), though they are both high affinity purine nucleobase transporters (Wallace, *et al.*, 2002). However, the architecture of the LmajNBT1 binding site appears to be very similar to that of *T. brucei* H2 (Figure 3.10*C*; Wallace, *et al.*, 2002). Both transporters bind their highest affinity substrate, hypoxanthine, through the same interactions with the purine ring and even the individual bond energies are very similar. The only

substantial difference between the two transporters appears to be in the binding of adenine, where a weak H-bond with the amine group is now formed with N1 instead. It is certainly possible that this reflects merely a minor shift of position of one amino acid residue in the binding site. Alternatively an H-bond acceptor such as aspartate may have been replaced by an H-bond donor such as serine.

A. *L. major* NBT1



B. hFNT1



C. *T. brucei* H2

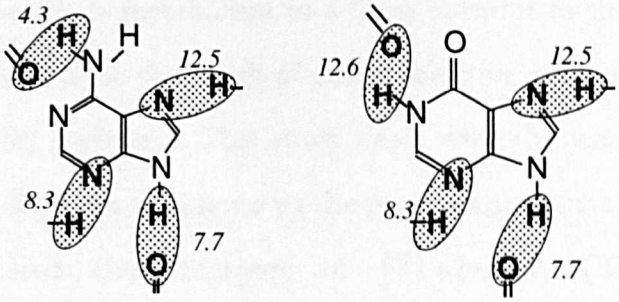


FIGURE 3.10 Model for the interactions between the LmajNBT1 (A), hFNT1 (B) and TbH2 (C) transporters and their permeants, adenine and hypoxanthine. Estimated Gibbs free energy for proposed bonds are indicated as – kJ/mol. The models for hFNT1 and TbH2 were adapted from (Wallace, *et al.* 2002).

The substrate recognition similarities of the two transporters extend to having far higher affinity for guanosine than for any other nucleoside, despite the fact that guanine is a lower affinity substrate than hypoxanthine. The only feasible explanation for this is that the optimal configuration for nucleoside binding is stabilised by an internal H-bond between the 5'hydroxyl and 2-NH2 groups. The fact that this confers higher affinity than for the other purine nucleosides shows just how similar the binding site environment of LmajNBT1 and TbH2 is. Table 3.3 lists the ΔG^0 and $\delta(\Delta G^0)$ of H2 alongside those of LmajNBT1.

3.3 Discussion

Purine nucleobase and nucleoside analogues are being used against a wide variety of diseases and infections (De Koning and Diallinas, 2000; Chen, *et al.*, 2002; De Clercq, 2002; Galmarini, *et al.*, 2002; El Kouni, 2003; De Clercq, 2004; Parker, *et al.*, 2004; De Clercq, *et al.*, 2005; Jordheim, *et al.*, 2005). The hypoxanthine analogue allopurinol has shown considerable promise for the treatment of Leishmaniasis, and several nucleoside analogues have shown anti-protozoal activity, but no rational approach to a purine-based chemotherapy for protozoan infections has been developed. Such an approach needs to take account of (1) efficient uptake of the analogue by the parasite and (2) conversion by the parasite's metabolism to a form harmful to the organism. Selective toxicity therefore needs to be the result of either selective uptake or differences in the metabolic enzymes for pathways. This study deals with the issues related to specific and efficient uptake of purine analogues by the protozoan parasite *Leishmania major*.

We have investigated the transport of [^{3}H]adenine, [^{3}H]hypoxanthine and [^{3}H]allopurinol and found that all three bases were taken up by the same transporter, LmajNBT1. This conclusion is based on (1) consistently monophasic inhibition profiles with Hill slopes of −1, leading to 100% inhibition of permeant uptake by the unlabelled inhibitor, (2) near identity of K_m values for hypoxanthine uptake and the K_i value for

hypoxanthine inhibition of adenine uptake (and the equivalent observations for [^{3}H]adenine and [^{3}H]allopurinol) and (3) internally consistent K_i and ΔG^0 values with a range of structural analogues, allowing the construction of a quantitative model for substrate recognition. The only previous report to date of nucleobase transport in *Leishmania*, by Hansen, *et al.* (1982), also found that hypoxanthine and adenine were probably transported by a single transporter in *Leishmania braziliensis panamensis*. In contrast, nucleoside transporters have been relatively extensively studied in *Leishmania donovani* (Vasudevan, *et al.*, 1998, 2001; Carter, *et al.*, 2000b; Ghosh and Mukherjee, 2000; Galazka, *et al.*, 2006) and found to display high affinity for their substrates, with K_m values typically between 0.3 and 5.0 μM. In view of the much higher K_i values for nucleosides reported here for LmajNBT1, it is unlikely that this transporter plays a significant physiological role in nucleoside salvage.

Allopurinol uptake has not previously been studied in *Leishmania* species. The discovery that, in contrast to *T. brucei* (De Koning and Jarvis, 1997b; Natto, *et al.*, 2005), allopurinol is taken up by only a single transporter, raises concerns about the ease with which resistance may develop, particularly since *Leishmania* species also express high affinity purine nucleoside transporters (Carter, *et al.*, 2000b; Vasudevan, *et al.*, 1998; Landfear, *et al.*, 2004; De Koning, *et al.*, 2005) and LmajNBT1 is thus unlikely to be an essential gene. It would therefore be prudent to use allopurinol mainly as part of combination chemotherapy.

Allopurinol displayed only a moderate affinity for LmajNBT1 and its maximal rate of uptake is at least 10-fold lower than for adenine or hypoxanthine, but it is clinically active against leishmaniasis at high doses. It could be speculated that other purine analogues, if salvaged more efficiently, could have a higher efficacy. We have therefore studied the substrate selectivity of LmajNBT1 in detail, constructing a quantitative model that allows predictions of the affinity of the transporter for potentially therapeutic analogues. While such models yield estimates of K_m rather than V_{max}, it

does allow rational design or selection of purine analogues that are likely to be accumulated efficiently inside the parasite.

Therapeutic action will then depend on such enzymes as the phosphoribosyltransferases, among others, that convert the analogues into nucleotides, though some purines, including kinase inhibitors, may not need any metabolic conversion for their mode of action. The purine metabolic pathways of *Leishmania* have been studied in detail (Hassan and Coombs, 1988; Berens, *et al.*, 1995) and most of the key enzymes have been characterised, cloned (Allen, *et al.*, 1995; Thiemann, *et al.*, 1998; Jardim, *et al.*, 1999; Sinha, *et al.*, 1999; Cui, *et al.*, 2001; Zarella-Boitz, *et al.*, 2004) and, in some cases, crystallised (Phillips, *et al.*, 1999; Shi, *et al.*, 1999), potentially allowing the design of specific inhibitors or subversive substrates. Compliance with the LmajNBT1 model would ensure selective and efficient salvage of such designer drugs. And since the substrate recognition models for the nucleobase transporters of *L. major* and *T. brucei* are almost identical, purine antimetabolites developed against either species may well be active against other members of the family Trypanosomatidae.

Relationships between transporters are usually defined by their degree of sequence homology, and transporters are classified into families on this basis. However, such classification provides limited information. The family of the Equilibrative Nucleoside Transporters (ENT) is a case in point. As reviewed by Hyde, *et al.* (2001), this family includes high affinity and low affinity transporters, equilibrative transporters and proton symporters, transporters that recognise only one or two specific nucleosides, and those that have broad specificity including nucleobases. It follows that only limited functional information can be gleaned from sequence similarities, and an additional classification based on substrate recognition would be of value. A striking illustration is provided by the adenosine transporters of *T. brucei*, P1 and P2, encoded by the genes *TbNT2* and *TbAT1*, respectively. Though the genes are closely related (57% identity at amino acid level) and both transport adenosine with similar affinity and rate, P1 also

transport guanosine and inosine, which are not substrates of P2 (Carter and Fairlamb, 1993; De Koning and Jarvis, 1999). Conversely, P2 efficiently transports adenine as well as trypanocidal drugs such as melaminophenyl arsenicals and diamidines (Barrett and Fairlamb, 1999; De Koning, 2001). These important pharmacological differences are readily explained by their proposed substrate binding models (De Koning and Jarvis, 1999; De Koning, *et al.*, 2005) but, at present, cannot be predicted from their primary sequences. Therefore, though TbAT1 and TbNT2 are genetic homologues, they are not functionally homologous.

After this work was published, Landfear's group has cloned and expressed the first gene for a *L. major* nucleobase transporter, designated *LmaNT3*. The LmaNT3 permease shows 33% identity to *L. donovani* nucleoside transporter 1.1 (LdNT1.1) and is proposed to be a member of the equilibrative nucleoside transporter (ENT) family (Sanchez, *et al.*, 2004b). This transporter is quite similar to the LmajNBT1 we characterised, as the functional expression studies in *Xenopus laevis* oocytes showed that LmaNT3 mediates high levels of uptake of hypoxanthine, xanthine, adenine and guanine. Moreover, LmaNT3 possess a high affinity for hypoxanthine, xanthine, adenine and guanine with K_m values of 16.5 ± 1.5, 8.5 ± 0.6, 8.5 ± 1.1 and 8.8 ± 4.0 μM, respectively. However, given that the order of affinities for the various substrates was reportedly different for LmaNT3 and LmajNBT1, the possibility that these are genuinely different transporter activities cannot be excluded. The fact that the two studies used different expression systems (promastigote and *Xenopus* oocytes) is a confounding factor and definitive conclusions cannot be drawn at this time.

The genes encoding LmajNBT1 and TbH2 have yet to be positively identified, but the current study clearly establishes them as functional homologues and speculates that the environment of the binding sites of the two transporters is very similar: their functional differences could be explained with a single amino acid substitution or even a slight positional shift in one residue. In contrast the best studied nucleobase

transporter in the human host, FNT1 (Wallace, *et al.*, 2002), and the *Toxoplasma gondii* hypoxanthine transporter TgNBT1 (De Koning, *et al.*, 2003) interact in an entirely different way with their permeants.

Chapter Four

NUCLEOBASE TRANSPORT IN *LEISHMANIA MEXICANA* AMASTIGOTES

4.1 Summary

Most of the currently available antileishmanial drugs have been discovered empirically, as until recently insufficient information was known about the biochemistry, physiology and molecular biology of these parasites and the interactions with their hosts (El Kouni, 2003). The limitations of the current treatment for leishmaniasis, as a result of drug resistance, the severe side effects of most of the existing therapeutic agents, and the urgent need for new therapeutic approaches, are well documented (Croft, 2001; Sundar, 2001a; Yardley and Croft, 2000; Leandro, *et al.*, 2003; Croft, *et al.*, 2006).

The development of a rational therapeutic strategy for the treatment and prevention of parasitic disease depends on exploitation of fundamental biochemical disparities between parasite and host, such as the inability of protozoan parasites to synthesize purines *de novo* (Berens, *et al.*, 1995). Current antiprotozoal agents often derive selectivity from selective accumulation by the parasite rather than the host cell (De Koning, 2001). The selectivity and the efficacy of purine antimetabolites can be achieved by the cell-surface transporters that mediate access to the cell, as substrate recognition by nucleobase transporters is strikingly different in humans and kinetoplastids such as *Trypanosoma brucei* (Wallace, *et al.*, 2002) and *Leishmania major* promastigotes (AlSalabi, *et al.*, 2003).

Purine and pyrimidine antimetabolites have been highly successful against many viral infections as well as malignancies (Kolb, 1997), and show great promise against protozoal infections as well (De Koning and Diallinas, 2000; El Kouni, 2003; De Koning, *et al.*, 2005). Allopurinol, a purine nucleobase analogue, is clinically used against various manifestations of Leishmaniasis (Das, *et al.*, 2001; Abrishami, *et al.*, 2002; Momeni, *et al.*, 2002).

Whereas purine transporters in promastigotes have now been studied in detail (Landfear, *et al.*, 2004; De Koning, *et al.*, 2005), very little is known about such transporters in the infective amastigote forms. The only study of purine transporters in amastigotes demonstrated that there are at least two high-affinity adenosine transporters in *L. donovani* amastigotes, T1 and T2 (Ghosh and Mukherjee, 2000). Purine nucleobase transport has yet to be described in this intracellular form, despite a clear interest from physiological and pharmacological perspectives.

In the previous chapter, we have shown that allopurinol is efficiently taken up by a high-affinity purine nucleobase transporter in *L. major* promastigotes, LmajNBT1 (Al Salabi, *et al.*, 2003). We here report the first identification and characterization of a purine nucleobase transporter in *Leishmania* amastigotes. Uptake of [^{3}H]hypoxanthine by *L. mexicana* amastigotes was mediated by a single high-affinity transporter, LmexNBT1, with a K_m of 1.6 ± 0.4 μM and high affinity for adenine, guanine and xanthine but low affinity for nucleosides and pyrimidine nucleobases. Allopurinol, an antileishmanial hypoxanthine analogue, was apparently taken up by the same transporter. Using [^{3}H]allopurinol, a K_m value of 33.6 ± 6.0 μM was obtained. All evidence was compatible with a model of a single purine nucleobase transporter being expressed in amastigotes. Using various purine nucleobase analogues, a model for the interactions between hypoxanthine and the transporter's permeant binding site was constructed. The binding interactions were compared with those of the LmajNBT1 transporter in *L. major* promastigotes and found to be very similar. This work has, in part, been previously published as Al Salabi et al. (2005) Antimicrob. Agents. Chemother. 49:3682-9 (see Appendix III).

4.2 Results

4.2.1 Purine transport in axenic amastigotes of *Leishmania*

Total uptake of adenosine in axenic amastigote forms of *L. mexicana* was determined using a rapid stop/oil spin protocol adapted from similar protocols with various other protozoa (De Koning and Jarvis, 1997a; De Koning and Jarvis, 1999; De Koning, *et al.*, 2000b; Al Salabi, *et al.*, 2003; De Koning, *et al.*, 2003; Chapter two). Figure 4.1 *A* demonstrates that the transport of 1 μM [^{3}H]adenosine transport was linear for at least 120 s at 0.021 ± 0.001 pmol(10^7 cells)$^{-1}$s^{-1} (linear regression; r^2 = 0.97). The addition of 1 mM unlabeled adenosine inhibited the uptake of 1 μM [^{3}H] adenosine by 87% (r^2 = 0.99), indicating that the vast majority of adenosine transport occurs via a mediated pathway (Figure 4.1*A*). These observations are consistent with the earlier report for adenosine transporter in amastigote forms of *Leishmania donovani* (Ghosh and Mukherjee, 2000).

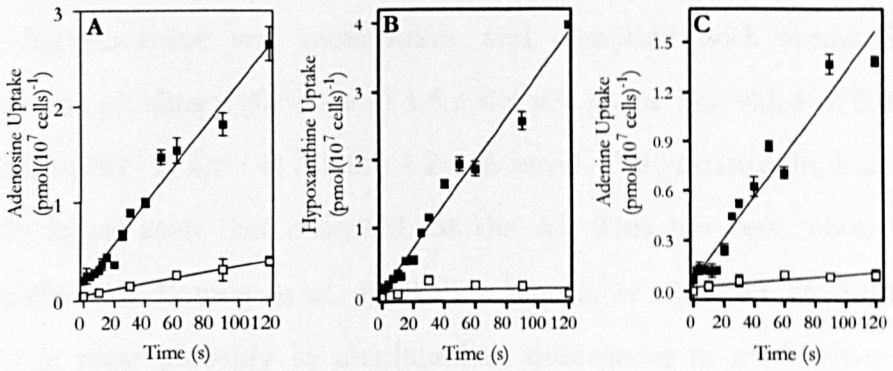


FIGURE 4.1 Linear purine transport in axenic amastigotes of *Leishmania mexicana*. **A.** Timecourse of 1 μM [^{3}H]adenosine uptake by *L. mexicana* amastigotes in the presence (□) or absence (■) of 1 mM unlabelled adenosine. **B.** Uptake of 1 μM [^{3}H]hypoxanthine was linear over 120 s (r^2 = 0.97) as calculated by linear regression, in the presence (□) or the absence (■) of 1 mM unlabelled hypoxanthine. **C.** Uptake of 1 μM [^{3}H]adenine was linear over 120 s. (r^2 = 0.94) as calculated by linear regression, in the presence (□) or the absence (■) of 1 mM unlabelled adenine. In some cases, an apparent positive intercept can be observed for the timecourses in the absence of inhibitor. This is most likely the caused by extracellular binding of radiolabel, which is displaced by the including of 1 mM non-radiolableled substrate.

Uptake of [^{3}H]hypoxanthine at 1 μM by the axenic amastigotes of *L. mexicana* was linear for up to 120 s (Figure 4.1*B*) and faster than [^{3}H]adenosine uptake, at 0.031 ± 0.001 pmol(10^7 cells)$^{-1}$s^{-1} (r^2 = 0.98). In the presence of 1 mM unlabelled hypoxanthine, [^{3}H]-hypoxanthine transport was not significantly different from zero (P = 0.7, F-test), indicating high affinity transport that is completely saturated at 1 mM. [^{3}H]adenine uptake (1 μM) was likewise linear over 120 s, and the least efficiently accumulated of the three purines tested at 1 μM of label, with a rate of 0.012 ± 0.001 pmol(10^7 cells)$^{-1}$s^{-1}, which was almost 95% inhibited by 1 mM unlabeled adenine (Figure 4.1*C*). Subsequent experiments were performed with [^{3}H]hypoxanthine using 60 s incubations, well within the linear phase of uptake and therefore measuring true initial rates of transport.

4.2.2 A high affinity purine nucleobase transporter in amastigotes

[^{3}H]Hypoxanthine transport (0.1 μM) measured in the presence of up to 1 mM of unlabelled hypoxanthine was monophasic and complied with simple Michaelis–Menten kinetics, yielding a K_m value of 1.6 ± 0.4 μM and a V_{max} value of 0.092 ± 0.057 pmol (10^7 parasites)$^{-1}$ s^{-1} (n = 4) (Figure 4.2 and *inset*). The variation in V_{max} values was considerably larger than that observed for the K_m. This has been observed also in previous studies (De Koning *et al.*, 1998; Burchmore, *et al.*, 2003; De Koning, *et al.*, 2003) and can most probably be attributed to differences in transporter expression levels under slightly different culture conditions (mid-log or late-log stage growth) (De Koning, *et al.*, 2000).

The transport of [^{3}H]Hypoxanthine was inhibited by a range of purine nucleobases and nucleosides (Table 4.1) but not sensitive to pyrimidines uracil, thymine, cytosine, and uridine, which inhibited 0.1 μM [^{3}H]hypoxanthine transport by just 27 ± 7%, 41 ± 11%, 41 ± 15%, and 16 ± 2%, respectively, at 1 mM (n =3). The hypoxanthine

transporter, designated *L. mexicana* nucleobase transporter 1 (LmexNBT1), appears to be mildly selective for oxopurines over aminopurines, judging from the K_i values for guanine and adenine (1.7 ± 0.1 and 4.2 ± 0.8 μM, respectively). None of the inhibition plots suggested the presence of more than one hypoxanthine transporter in *L. mexicana* amastigotes, as Hill slopes were consistently near –1 and complete inhibition to control values (transport at 0 °C, 0 s) was generally observed.

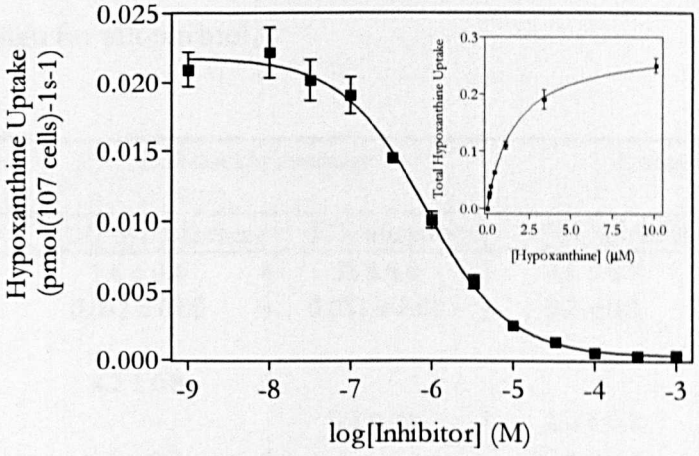


FIGURE 4.2 Transport of 0.1 μM [^{3}H]hypoxanthine over 60 s was inhibited by the indicated concentrations of unlabelled hypoxanthine with an IC_{50} value of 0.81 μM for this experiment. The inset depicts the conversion of the hypoxanthine inhibition data to a Michaelis-Menten plot of total hypoxanthine uptake, with a K_m value of 1.6 μM and a V_{max} of 0.28 pmol(10^7 cells)$^{-1}$s^{-1} for this experiment.

4.2.3 [^{3}H]allopurinol transport by *L. mexicana* amastigotes

Allopurinol is a close structural analogue of hypoxanthine and in *L. major* promastigotes is indeed accumulated through the hypoxanthine transporter LmajNBT1 (Al Salabi, *et al.*, 2003). We therefore investigated whether a similar situation exists in amastigotes. As *L. major* amastigotes cannot be kept in axenic culture, we used

amastigotes from *L. mexicana*. Using these cells, transport of allopurinol was clearly less efficient than of hypoxanthine, with a rate for 5 μM [^{3}H]allopurinol of 0.0061 ± 0.0002 pmol(10^7 cells)$^{-1}$s^{-1} (linear regression of 6 points over 4 minutes; r^2 = 0.99), which was completely inhibited by 1 mM hypoxanthine (uptake not significantly different from zero; P = 0.33, F-test). Transport of [^{3}H]allopurinol (5 μM) was still detectable, however, in the presence of 1 mM unlabelled allopurinol (0.00052 ± 0.00014 pmol(10^7 cells)$^{-1}$s^{-1} (linear regression of 5 points over 10 minutes; P = 0.03, F-test), though greatly reduced. These results indicate that the allopurinol transporter has a higher affinity for hypoxanthine than for allopurinol.

	L. mexicana amastigotes				*L. major* promastigotes			
	[^{3}H]-hypoxanthine		[^{3}H]-allopurinol		[^{3}H]-adenine		[^{3}H]-allopurinol	
K_m[a]	1.6 ± 0.4	4	33 ± 6.0	3	4.6 ± 0.9	3	54 ± 2.9	3
V_{max}[b]	0.092 ± 0.05	4	0.051 ± 0.01	3	3.2 ± 0.3	3	0.24 ± 0.06	3
Adenine[1]	4.2 ± 0.8	5						
Hypoxanthine[1]			3.8 ± 0.6	3	1.3 ± 0.3	3	0.30 ± 0.09	3
Guanine[1]	1.7 ± 0.1	4			2.8 ± 0.7	4		
Xanthine[1]	13 ± 2	3			23 ± 8	3		
Allopurinol[1]	39 ± 6	3			56 ± 1.5	3		
Aminopurinol[2]	170 ± 20	4			ND			
Purine[1]	3.4 ± 0.2	3			6.7 ± 0.4	3		
1-Deazapurine[2]	54 ± 5	3			26 ± 4.1	3		
3-Deazaguanine	130 ± 14	3			48 ± 5.0	3		
6-Thioguanine[1]	10 ± 1.2	3			6.2 ± 0.8	3		
7-Deazaguanine	>1000	3			430 ± 140	3		
9- Deazaguanine	204 ± 28	4			204 ± 4	3		
Adenosine[1]	950 ± 240	3			5150 ± 550	3		
Inosine[1]	380 ± 26	3			125 ± 15	3		
Guanosine[1]	210 ± 48	3			68 ± 17	4		

TABLE 4.1 Kinetic constants of purine nucleobase uptake in *L. mexicana* amastigotes and *L. major* promastigotes. Kinetic parameters were determined through competitive inhibition of [^{3}H]-hypoxanthine (amastigotes) or [^{3}H]adenine (promastigotes). In a few cases, extrapolation was required due to limitations of solubility of the inhibitor and based on the assumption of a Hill slope of −1 and eventual 100% inhibition. Extrapolation was not attempted when inhibition at the highest inhibitor concentration was < 50%. Permeant concentration was 2 μM (allopurinol) or 0.1 – 0.5 μM (hypoxanthine), except for determinations of K_m. (data for *L. major* promastigotes was taken from (Chapter three). [a] expressed in μM, [b] expressed as pmol(10^7 cells)$^{-1}$s^{-1}.

Competition experiments showed that transport of 2 μM [^{3}H]allopurinol over 120 s was indeed inhibited more strongly by hypoxanthine than by allopurinol (Figure 4.3*A*), with a K_i value of 3.8 ± 0.6 μM for hypoxanthine and a K_m of 33 ± 6.0 μM for allopurinol (n = 3)(Figure 4.3*B*). These data are all consistent with a single high-affinity transporter for purine nucleobases including allopurinol. While the V_{max} values of LmexNBT1 for hypoxanthine and allopurinol are similar (Table 4.1), the efficiency of uptake, expressed as K_m/V_{max}, is considerably higher for hypoxanthine.

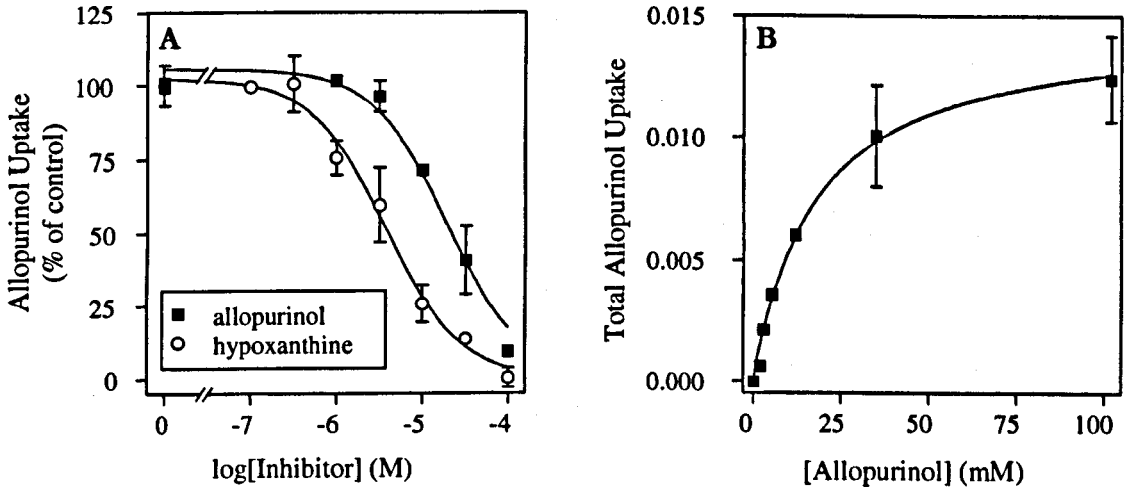


FIGURE 4.3 Transport of [^{3}H]allopurinol by *L. mexicana* amastigotes. A. Inhibition of 2 μM [^{3}H]allopurinol transport over 120 s by up to 100 μM of unlabelled allopurinol (■) or hypoxanthine (○). B. Michaelis-Menten plot of allopurinol uptake, derived from the data in frame A.

4.2.4 Structure-activity relationships of purine analogues and the LmexNBT1

The inhibitory effects of purine analogues for LmexNBT1 were tested by measuring initial uptake rates of 0.1 μM [^{3}H]hypoxanthine in the presence of varying concentrations of the test compound. Results were plotted as log inhibitor concentration versus hypoxanthine uptake and K_i values, calculated from the IC_{50}

values, were used to calculate the estimated Gibbs free energy of binding ΔG^0 (reviewed by De Koning, *et al.*, 2005). The results, summarized in tables 4.1 and 4.2, show that the LmexNBT1 transporter shows broad specificity for purine nucleobases but displayed low affinity for the corresponding nucleosides.

LmexNBT1 displayed equal affinity for guanine (Figure 4.4*A*) and hypoxanthine (Figure 4.2), showing that the 2-amino group of guanine did not contribute to binding, nor posed a steric hindrance to binding. However, affinity for xanthine (Figure 4.4*A*) was markedly reduced ($\delta(\Delta G^0)$ = 5.5 kJ/mol compared to hypoxanthine). As this is unlikely to be the result of steric hindrance at position 2, this must be the result of protonation of the pyrimidine nitrogen at position 3 of the purine ring that occurs in xanthine.

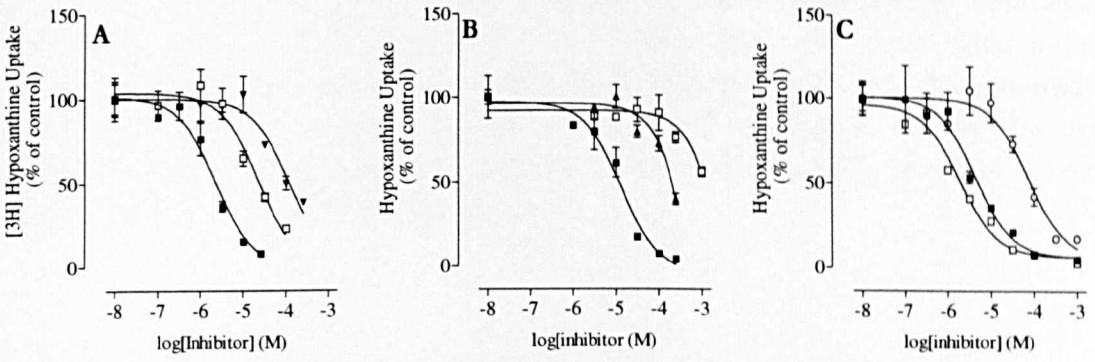


FIGURE 4.4 Inhibition of 0.1 μM [^{3}H]hypoxanthine uptake in axenic amastigotes of *L. mexicana* by **A**: guanine (■), xanthine (□) and 3-deazaguanine (▼). **B**: 6-thioguanine (■), 7-deazaguanine (□) and 9-deazaguanine (▲). **C**: Purine (■), adenine (□) and 1-deazapurine (○). Data were expressed as percent of control being uptake in the absence of any inhibitor, typically 0.005 – 0.02 pmol/10^7 cells/s), showing total hypoxanthine transport as opposed to [^{3}H]hypoxanthine transport only.

A comparison between guanine and 3-deazaguanine (Figure 4.4*A*) shows that the unprotonated N3 contributes 11.2 kJ/mol to the binding energy of guanine (Table 4.2). As only 5.5 kJ/mol is lost in xanthine it can be postulated that weak hydrogen bonds

Compound	$\Delta(G^0)$	$\delta(\Delta G^0)$	Control
Hypoxanthine	-34.4		
Adenine	-31.9	2.5	Hypoxanthine
Allopurinol	-26.2	8.2	Hypoxanthine
Aminopurinol	-22.3	9.6	Adenine
Xanthine	-28.9	5.5	Hypoxanthine
Guanine	-34.2	0.2	Hypoxanthine
Inosine	-20.3	14.1	Hypoxanthine
Adenosine	-17.9	14.0	Adenine
Guanosine	-21.9	12.3	Guanine
Purine	-32.5	-0.6	Adenine
1-Deazapurine	-25.3	7.2	Purine
3-Deazaguanine	-23.0	11.2	Guanine
6-Thioguanine	-29.7	4.5	Guanine
7-Deazaguanine	>-18	17.2	Guanine
9-Deazaguanine	-21.9	12.3	Guanine

TABLE 4.2 Gibbs free energies (kJ/mol) of substrate interacting with LmexNBT1 transporter. Gibbs free energy of substrate-transporter interactions was calculated from the K_m and K_i values listed in Table 1, using the Nernst equation as described (De Koning and Jarvis, 1999; Wallace, et al., 2002). The difference with a control compound, either hypoxanthine as the highest affinity compound, the corresponding physiological nucleobase (in the case of chemical analogues) or (in the case of nucleosides) the corresponding nucleobase yielded the $\delta(\Delta G^0)$, the loss in binding energy relative to the control compound.

totalling ~ 6 kJ/mol can be established between the LmexNBT1 binding pocket and N(3)H and/or the 2-keto group of xanthine. From inhibition plots with 9-deazaguanine (Figure 4.4*B*), the Gibbs free energy of H-bonds with N(9)H was similarly estimated at –12.3 (Table 4.2). The K_i-value for 7-deazaguanine could not be clearly determined, due to very low affinity and solubility limitations, but is shown to be over 1 mM, which would indicate a $\delta(\Delta G^0) > 15$ kJ/mol for N(7). Furthermore, the substitution of the 6-keto group for a thione group (6-thioguanine) reduced the ΔG^0 by 4.5 kJ/mol (6-thioguanine vs guanine; Figure 4.4*B*). This could be an indication of a hydrogen bond interacting with the keto group, as thiones are much weaker H-bond acceptors (Pitha, *et al.*, 1975), though the possibility of a steric effect by the larger sulfur atom provides an alternative explanation. Indeed, the $\delta(\Delta G^0)$ between hypoxanthine and purine,

which lacks both the 6-keto and lactam hydrogen on N1, is only 1.9 kJ/mol (Table 4.2). However, this needs to take into account the strong hydrogen bond with the pyrimidine N1, estimated at 7.2 kJ/mol from the comparison between purine and 1-deazapurine (Figure 4.4*C*), bringing the actual contribution of the 6-keto/N(1)H combination to 9.1 kJ/mol of which 4.5 kJ/mol could be tentatively assigned to the keto group from the $\delta(\Delta G^0)$ of 6-thioguanine. Finally, adenine (Figure 4.4*C*) and purine proved to have the same affinity for LmexNBT1 (Table 4.1), confirming the preference for a hydrogen bond acceptor at position 6 of the purine ring. The above observations were combined into a model for the binding of purine substrates by LmexNBT1 and compared to the model for substrate binding by the equivalent transporter of *L. major* (Figure 4.5).

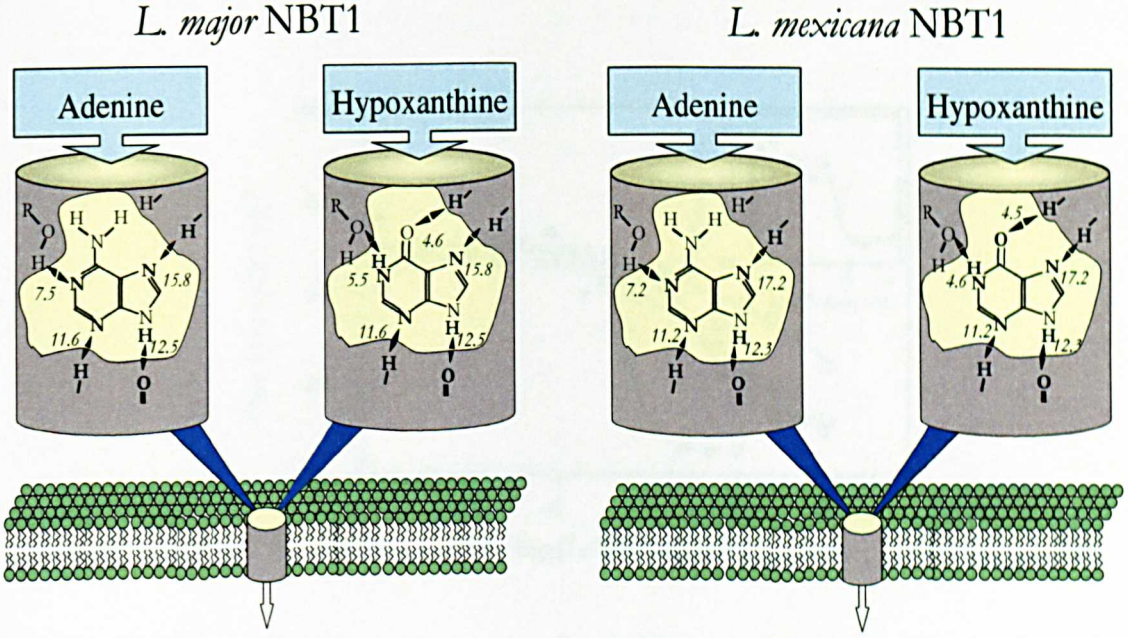


FIGURE 4.5 Schematic representation of the LmajNBT1 (A) and LmexNBT1 (B) transporters and the interactions with their permeants, adenine and hypoxanthine. Estimated Gibbs free energy for proposed bonds are indicated as – kJ/mol. The model for LmajNBT1 was adapted from (Al Salabi, *et al.*, 2003).

4.2.5 Antileishmanial activity of some nucleobases analogues

Allopurinol is well known to possess antileishmanial activity but we previously noted that, at least in promastigotes, aminopurinol and the pyrimidine nucleobase 5-fluorouracil displayed substantially higher activity (Papageorgiou, *et al.*, 2005). We therefore tested the same compounds on the human-infective amastigotes stage, using pentamidine as positive control and the same technique based on the fluorophore Alamar Blue (Figure 4.6). The method was validated using cell counts in parallel to the fluorescence assay, yielding the same IC_{50} values (Figure 4.6, *inset*). As with *L. major* promastigotes, aminopurinol and 5-fluorouracil were 10-20-fold more active against axenic *L. mexicana* amastigotes than allopurinol. IC_{50} values (μM, $n = 3$) were 5.6 ± 1.0, 630 ± 87, 65 ± 5 and 36 ± 6 for pentamidine, allopurinol, aminopurinol and 5-fluorouracil, respectively.

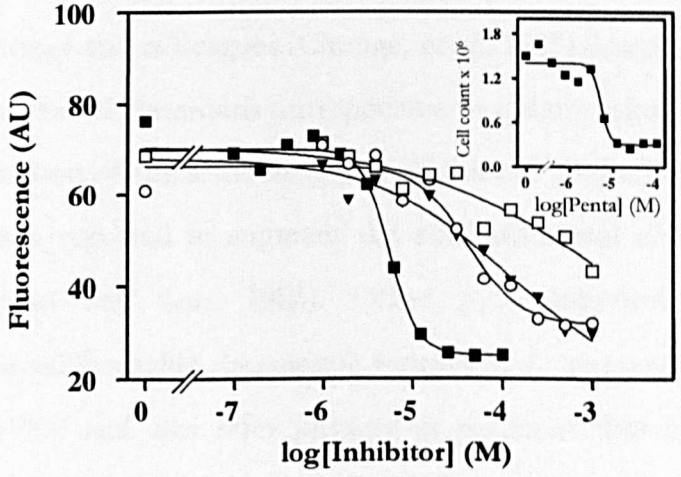


FIGURE 4.6 Effect of various drugs on *L. mexicana* amastigotes in axenic culture. Amastigotes (2×10^5) were incubated with doubling dilutions of pentamidine (■), allopurinol (□), aminopurinol (▼) and 5-fluorouracil (○) for 72 h and for an additional 48 h in the presence of Alamar Blue reagent as described in Chapter two. ED_{50} values for pentamidine determined by fluorescence or cell counts (*inset*) were identical.

4.3 Discussion

Purine and pyrimidine antimetabolites are widely used to combat a variety of infectious diseases and other pathologies (De Koning and Diallinas, 2000). Current evidence indicates that purine analogues alone or in combination with other chemotherapeutic agents are very effective against almost any fast growing cells (El Farrash, *et al.*, 2003; Namazi, 2004). Allopurinol, a purine analogue, has been used to treat leishmaniasis alone (Pfaller and Marr, 1974; Marsden, *et al.*, 1984) or combined with other drugs (Martinez and Marr, 1992; Martinez, *et al.*, 1997; Denerolle and Bourdoiseau, 1999; Momeni, *et al.*, 2002; Pasa, *et al.*, 2005). It should be pointed out, however, that the efficacy of allopurinol monotherapy remains unclear. A large-scale WHO-sponsored randomized trial showed no difference between allopurinol and placebo-treated groups (Velez, *et al.*, 1997) and it may be that the value of allopurinol in the treatment of leishmaniasis is solely by synergistically enhancing the action of other treatments and there is much, though largely anecdotal evidence for this. One such report, by Chunge and colleagues (Chunge, *et al.*, 1985) describes the treatment of 5 patients with visceral leishmaniasis unresponsive to sodium stibogluconate that were cured by a combination of the same drug plus additional allopurinol. *In vitro* as well, allopurinol has been reported to augment the antileishmanial effects of pentavalent antimonials (Berman and Lee, 1983). Other pyrazolopyrimidines such as 9-deazainosine displayed favorable therapeutic indexes in *L. donovani*-infected hamsters (Berman, *et al.*, 1987) and also offer promise as potential chemotherapeutic agents (Marr, *et al.*, 1984). However, it is widely acknowledged that the potential for damaging side effects can pose a challenge to the development of purine-based drugs (El Kouni, 2003).

The selectivity and efficacy of purine antimetabolites can be achieved through the enzymes of the purine metabolic pathways that convert the pro-drug to the cytotoxic metabolite, usually a nucleotide analogue, and understanding substrate specificity of the enzymes involved is important in both drug efficacy and selectivity. The

metabolism of allopurinol in *Leishmania* species to the active metabolite 4-aminopyrazolo(3,4-*d*)pyrimidine ribonucleoside triphosphate, which is incorporated into RNA, has been well studied (Marr, *et al.*, 1978; Nelson, *et al.*, 1979a; Marr, 1983; Marr and Berens 1983). The drug is selective because mammalian cells do not show this conversion or incorporation (Nelson, *et al.*, 1973).

In addition, many antiprotozoal drugs achieve their selectivity through uptake by cell-surface transporters on the target cell rather than the host's cells (De Koning, 2001). The main human nucleobase transporter, hFNT1, displays very low affinity for allopurinol (Razavi, *et al.*, 1993; Wallace, *et al.*, 2002), whereas allopurinol uptake in *Trypanosoma brucei* was mediated by high affinity nucleobase transporters (De Koning and Jarvis, 1997a; Natto, *et al.*, 2005). We have demonstrated that a strategy of purine-based chemotherapy, designed for specific uptake by protozoan parasites, is feasible and depends on detailed knowledge of the substrate recognition motifs of the respective transporters (Wallace, *et al.*, 2002; Wallace, *et al.*, 2004; De Koning, *et al.*, 2005).

The characterizations of [^{3}H]hypoxanthine, [^{3}H]allopurinol and [^{3}H]uracil transports in *L. major* promastigotes have recently been reported (Chapter two; Papageorgiou, *et al.*, 2005), but the presence of nucleobase transporters in amastigotes has not yet been reported. The issue is important in understanding the selectivity of the drug as well as the potential for the development of resistance as allopurinol-resistant *Leishmania* lines have been described (Cavaliero, *et al.*, 1999; Kamau, *et al.*, 2000) and appears to be easily inducible. We consider that this resistance might be related to loss of transporter function as allopurinol was taken up by a single nucleobase transporter in *L. major* promastigotes (Al Salabi, *et al.*, 2003), in sharp contrast to the situation in *T. b. brucei*, where the presence of multiple allopurinol transporters appears to preclude transport-related resistance (Natto, *et al.*, 2005). We have therefore conducted a study of purine nucleobase uptake in *Leishmania mexicana* amastigotes, with particular emphasis on allopurinol uptake. We found that hypoxanthine and allopurinol are taken up by a

single plasma membrane transporter, LmexNBT1, and characterized its substrate profile in detail.

LmexNBT1 proved extremely similar to LmajNBT1. A comparison of their substrate binding models (Figure 4.5) reveals that the positions and even the relative strengths of the hydrogen bonds are almost identical between the two transporters, suggesting a very similar architecture for the two transporters. This level of conservation is remarkable in that it signifies conservation both throughout the lifecycle and between *Leishmania* species from different continents. Furthermore, the binding site architecture of these transporters appears to be very similar to that of the H2 hypoxanthine/allopurinol transporter in bloodstream *T. b. brucei*, but very different from the binding motif of hFNT1 (Al Salabi, *et al.*, 2003; Wallace, *et al.*, 2004). This suggests that these related kinetoplastids would accumulate similar purine antimetabolites, which could be designed to be excluded from human cells. At this moment, none of the genes for these transporters involved have been identified with any certainty, precluding an assessment of whether the high level of functional conservation between the various *T. brucei* and *Leishmania* transporters are matched by genetic conservation. However, all protozoan purine transporter genes cloned to date were members of the Equilibrative Nucleoside Transporter family (De Koning, *et al.*, 2005) and the percentage identity between the *T. b. brucei* TbNBT1/H4 or TbNT8.1 nucleobase transporters and the *L. major* LmNT3 nucleobase transporter was only 50% (Burchmore, *et al.*, 2003; Henriques, *et al.*, 2003; Sanchez, *et al.*, 2004b).

LmexNBT1 showed only moderate affinity for allopurinol and aminopurinol. Yet, it seems unlikely that transport rates are a limiting factor in the efficacy of pyrazolopyrimidines against leishmaniasis, the conversion to the aminopurinol riboside triphosphate being a more likely bottleneck: aminopurinol, despite having lower affinity for LmexNBT1, was tenfold more active against axenic amastigotes in culture. Nelson and coworkers reported the direct incorporation of aminopurinol into the aminopurine nucleotide pool, and subsequently into the RNA, of both *Leishmania* and

T. cruzi (Nelson, *et al.*, 1973b; Marr, *et al.*, 1978; Nelson, *et al.*, 1979a) by means of phosphoribosylation – a conversion that does not happen in humans (Nelson, *et al.*, 1973).

This begs the question as to why aminopurinol has not been used against leishmaniasis, particularly as it has been long known that aminopurinol is more potent than allopurinol against *Leishmania* promastigotes *in vitro* (Avila, *et al.*, 1982) as well as *T. cruzi* in mice (Avila, *et al.*, 1983). In mammals, aminopurinol is used as an experimental drug, which reduces the release of lipoproteins by the liver, lowering plasma cholesterol levels (Shiff, *et al.*, 1971). Chronic use, however, induces a 'fatty liver' (Murakoshi, *et al.*, 1985) and adrenal cell hypertrophy by depleting adrenal cholesterol (Kovanen, *et al.*, 1980; Mazzocchi, *et al.*, 1988). Aminopurinol given to mice for 10 days at 1 mg/kg induced no toxic effect, but an increase to 10 mg/kg caused high mortality rates and hepatomegaly (Avila, *et al.*, 1983). A toxicological study in rodents, dogs and cats found tolerance to high single doses of aminopurinol but mouse LD_{50} values of 25 mg/kg when 5 consecutive daily doses were given (Philips, *et al.*, 1956). The hepatotoxic effects lead to the abandonment of aminopurinol as a potential anti-leukemia drug (Shaw, *et al.*, 1960), but levels active against *T. cruzi* in mice (Avila, *et al.*, 1983) were found to be non-toxic to humans (Shaw, *et al.*, 1960).

In summary, we have undertaken the first study of nucleobase transport in *Leishmania* amastigotes and identified a high affinity nucleobase transporter that is responsible for the uptake of allopurinol and other purine bases. As the evidence is consistent with a model for a single hypoxanthine/allopurinol transporter, the potential for the rapid development of allopurinol resistance by loss of such a transporter would appear to be a potential concern. However, the use of purine analogues in combination chemotherapy with another antileishmanial may well be synergistic and prevent or even overcome early onset of resistance to either drug.

Chapter Five

THE SUBSTRATE RECOGNITION MOTIF OF THE H4 NUCLEOBASE TRANSPORTER IN *TRYPANOSOMA BRUCEI* PROCYCLICS EXPLAINS ITS BROAD SPECIFICITY

5.1 Summary

The mechanism of purine nucleobase transport in bloodstream forms of the protozoan parasite *Trypanosoma brucei brucei* was previously investigated, and two different hypoxanthine transporters, H2 and H3, were identified. It was found that these distinct systems had different affinities and substrate specificities. The H2 transporter displayed a much higher affinity and a broader specificity than H3 (respective apparent K_m values of 123 ± 15 nM and 4.7 ± 0.9 μM , and V_{max} values of 1.1 ± 0.2 and 1.1 ± 0.1 pmol(10^7 cells)$^{-1}$ s^{-1}, respectively. For instance, the H2 transporter was inhibited by purine nucleobases and analogues as well as by some pyrimidine nucleobases such as uracil and one nucleoside guanosine, whereas H3 was not inhibited by any nucleosides or pyrimidines (De Koning and Jarvis, 1997b).

Prior to the findings of this study, purine nucleobase transport in procyclic *T. b. brucei* had been studied, and it was thought that only one distinct hypoxanthine transporter, named H1, was expressed in this stage of the life cycle. H1 displayed K_m and V_{max} values of 9.3 ± 2.0 μM and 4.5 ± 0.8 pmol(10^7 cells)$^{-1}$ s^{-1}, respectively and possessed high affinity for all natural purine nucleobases such as adenine, guanine, and xanthine as well as the purine analogue allopurinol with *Ki* values of 3.6 μM, 1.8 μM, 7.2 μM, and 5.0 μM respectively. This transporter was not inhibited by nucleosides and pyrimidine nucleobases (De Koning and Jarvis, 1997a).

A recent study from our group has led to the identification of the gene *TbNBT1* from *Trypanosoma brucei brucei.* This gene encodes a 435-residue protein of the Equilibrative Nucleoside Transporter family (Burchmore, *et al.*, 2003). The gene was expressed in both the procyclic and bloodstream forms of *Trypanosoma brucei.* Expression of TbNBT1 in a *Saccharomyces cerevisiae* strain lacking an endogenous purine transporter allowed growth on adenine as sole purine source and introduced a high affinity transport activity for adenine and hypoxanthine, with K_m values of 2.1 ±

0.6 and 0.66 ± 0.22 μM, respectively, as well as high affinity for xanthine, guanine, guanosine, and allopurinol and moderate affinity for inosine.

As mentioned above, the H1 was thought to be the only nucleobase transporter present in procyclic forms of *Trypanosoma brucei*. However, the nucleobase transporter characterized in yeast expressing TbNBT1 possessed > 10-fold higher affinity than H1 and was expressed in procyclics. Thus, there was a need to re-investigate the hypoxanthine uptake systems using very low concentrations of [^{3}H]hypoxanthine in procyclic forms of *Trypanosoma brucei*. Using these conditions, we detected a previously unknown high affinity transport activity, which we designated H4, and reconfirmed the presence of the lower affinity H1 transporter.

In this chapter, we report the identification and characterization of this novel nucleobase transporter *Trypanosoma brucei* procyclic forms. The kinetics profile obtained from the characterization of H4 was identical to the one observed in parallel from the yeast-expressed nucleobase transporter TbNBT1. Moreover, introduction of tetracycline-inducible RNAi constructs in procyclic trypanosomes, reduced H4-activity by ~90%. These finding confirmed that the *TbNBT1* gene encodes the first high affinity nucleobase transporter from protozoa to be identified at the molecular level and that H4 is likely to be the gene product of *TbNBT1*.

5.2 Results

5.2.1 Characterization of two hypoxanthine transporters in procyclic *T. brucei*

Total uptake of a final concentration of 30 nM [^{3}H]hypoxanthine in procyclic forms of *Trypanosoma brucei* was examined using a rapid-stop oil-spin technique (De Koning and Jarvis, 1997a). All experiments were performed using an incubation time of 30 s, within the linear phase of uptake and therefore measuring true initial rates of

transport. The uptake of 30 nM [^{3}H]hypoxanthine measured in the presence of 0-1000 μM of unlabeled hypoxanthine revealed the presence of two distinct hypoxanthine transporters in *T. b. brucei* procyclic cells (Figure 5.1). A previously reported lower affinity transporter, H1 (De Koning and Jarvis, 1997a), was observed alongside a higher affinity hypoxanthine transport activity, which we designated H4. In the current set of experiments, conducted at 30 nM [^{3}H]hypoxanthine, H1 and H4 activities were separated by fitting inhibition plots to an equation for two-site competition (Figure 5.1*A*) and to Eadie-Hofstee plots (Figure 5.1*B*), which produced kinetic constants in good in agreement with each other. Under these conditions, the kinetic constants for H1 were very similar to those previously reported (De Koning and Jarvis, 1997a). Figure 5.1 also shows that the uptake was saturable, with K_m and V_{max} values of 15.2 ± 2.3 μM and 2.4 ± 0.7 pmol(10^7 cells)$^{-1}$ s^{-1}, respectively, for the lower affinity H1 component (mean values from seven separate experiments). Although the H4 transporter has a lower V_{max} (0.27 ± 0.08 pmol (10^7 cells)$^{-1}$ s^{-1} (n = 7)), it was generally responsible for >85% of [^{3}H]hypoxanthine transport at 30 nM of label, due to its much higher affinity for hypoxanthine (K_m = 0.55 ± 0.07 μM (n = 7).

To further investigate the presence of the two transporters, inhibition experiments were performed on the uptake of 30 nM [^{3}H]hypoxanthine in procyclic forms of *Trypanosoma brucei* to study the selective inhibitors of H4. Uracil and guanosine do not inhibit H1-mediated [^{3}H]hypoxanthine transport up to 1 mM (De Koning and Jarvis, 1997a), but dose-dependently inhibited H4 activity (Figure 5.1*A*). However, affinities for adenine, guanine, xanthine, and allopurinol were similar for H1 and H4 (Table 5.1 and De Koning and Jarvis, 1997a). The K_m value of H4 was also significantly higher than previously reported for the *T. b. brucei* H2 transporter characterized in bloodstream forms (De Koning and Jarvis, 1997b) (H4 = 0.55 ± 0.07 μM and H2 = 0.12 ± 0.01 μM; $p < 0.01$, unpaired Student's t test). In addition, affinity for guanine was lower and for inosine higher for H4 than for H2 ($p < 0.05$). Most significantly, however, substitutions of N3 or N7 in guanine for CH (3-deaza- and 7-deazaguanine) have been

shown to reduce affinity for H2 by 28- and 150- fold, respectively (Wallace, *et al.*, 2002), but had little or no effect on affinity for H4 (Figure 5.2 and Table 5.2).

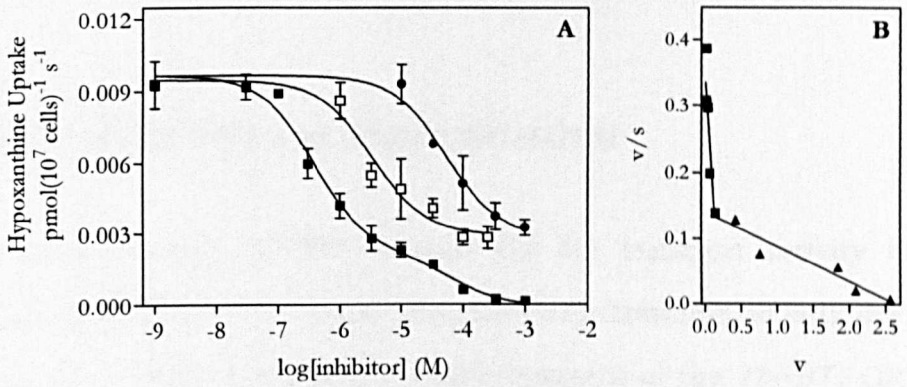


FIGURE 5.1 Hypoxanthine transport in Procyclic cells of strain 427 of *T. b. brucei*. *A* shows the uptake of 30 nM [^{3}H]hypoxanthine for 30 s, in the presence or absence of various concentrations of unlabeled hypoxanthine (■), uracil (●), or guanosine (□). Only the plot with hypoxanthine fitted a two-component inhibition model significantly better than a model for a single transporter ($p = 0.02$, F test). *B* shows the data with unlabeled hypoxanthine converted to an Eadie-Hofstee plot, showing two transporter activities with K_m values of 0.64 and 18.8 μM, respectively, and V_{max} values of 0.22 and 2.7 pmol(10^7 cells)$^{-1}$ s^{-1}, respectively. v, rate of [^{3}H]hypoxanthine transport; [s], permeant concentration.

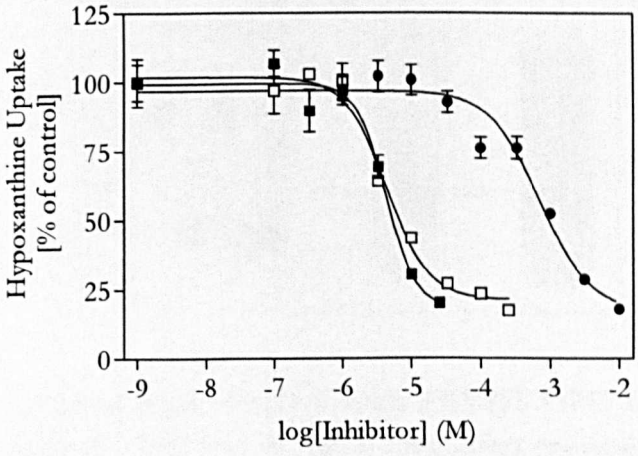


FIGURE 5.2 Inhibition of 100 nM [^{3}H]hypoxanthine uptake in Procyclic cells of strain 427 of *T. b. brucei* by increasing concentrations of unlabeled guanine (■: IC_{50} = 4.2 μM), 3-deazaguanine (□; IC_{50} = 4.3 μM), or adenosine (●; IC_{50} = 714 μM), and expressed as percentage of control (no inhibitor).

These results establish that H4 is a previously unknown transporter, distinct from either H1 or H2, and indeed from H3, which is a relatively low affinity hypoxanthine transporter in *T. b. brucei* bloodstream forms (De Koning and Jarvis, 1997b).

5.2.2 Effect of *TbNBT1* RNAi on H4 transport activity

Further evidence that *TbNBT1* encodes the H4 transport activity derives from experiments with RNAi. *T. b. brucei* procyclics were transformed with the vector p2T7 (LaCount, *et al.*, 2000), containing a 0.6-kb fragment of the *TbNBT1* ORF under the control of the tetracycline-inducible promotor. Northern blots revealed that *TbNBT1* mRNA levels in these cells were ~ 80% reduced after 5 days incubation with 1 μg/ml tetracycline (Figure 5.3; Burchmore, *et al.*, 2003). Incubation with tetracycline also greatly reduced the transport rate of the high affinity hypoxanthine transporter, and while the K_m did not significantly differ between tetracycline exposed and control cells (0.24 ± 0.06 *versus* 0.17 ± 0.04 μM), the V_{max} was reduced from 3.2 ± 0.2 to 0.20 ± 0.04 pmol $(10^7 \text{ cells})^{-1}$ s^{-1} (Figure 5.4).

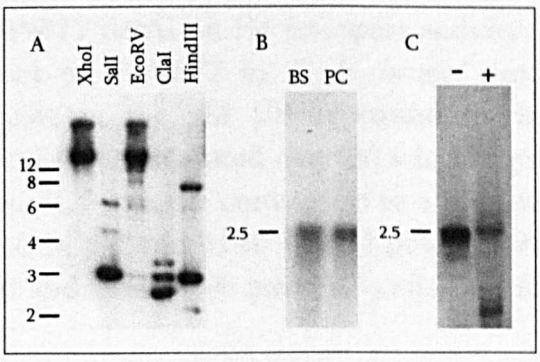


FIGURE 5.3 High stringency hybridisation with *TbNBT1* ORF. Genomic DNA of *T. b. brucei* strain 927, digested with the indicated restriction enzymes (*A*), total *T. b. brucei* RNA (*B*) or total RNA from *T. b. brucei* strain 29-13, transformed with p2T7TbNBT1 (*C*) was isolated and hybridised with the *TbNT1* ORF. Fragment sizes are indicated in kb. The blots were washed with 0.1 x SSC at 60 °C. BS, bloodstream forms; PC, procyclics. Reproduced from Burchmore et al. (2003).

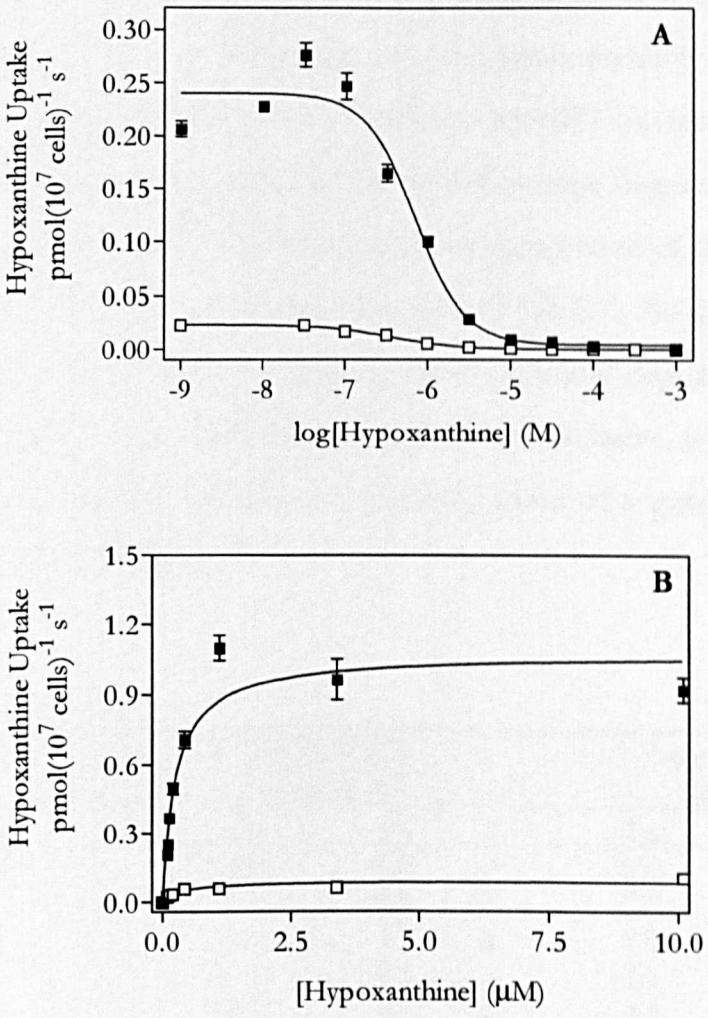


FIGURE 5.4 Effect of *TbNBT1* RNAi on H4 transport activity. Expression of a double-stranded RNAi fragment of *TbNBT1* in *T. b. brucei* procyclics was induced by tetracycline. *A.* Transport of 0.1 μM [^{3}H]hypoxanthine in the induced (□) and uninduced control (■) cells was measured over 30 s in the presence of 0–1000 μM of unlabeled hypoxanthine. *B.* shows the conversion to a Michaelis-Menten plot. K_m and V_{max} values were 0.34 ± 0.14 μM and 0.090 ± 0.011 pmol(10^7 cells)$^{-1}$ s^{-1} for the induced cells and 0.25 ± 0.07 μM and 1.07 ± 0.08 pmol(10^7 cells)$^{-1}$ s^{-1} for the uninduced control cells.

5.2.3 Kinetic parameters of *TbNBT1* in yeast and H4 in procyclic

The results described in the previous sections led to the working hypothesis that the *TbNBT1* gene encodes the H4 nucleobase transport activity. It must be pointed out,

however, that the RNAi result is not entirely conclusive, as it has become clear, on completion of the *T. b. brucei* genome, that the genes probably encoding the other purine nucleobase transporters are very similar to TbNBT1 (reviewed in De Koning, *et al.*, 2005) and could have been affected by the rather large fragment used in the NBT1 RNAi experiment. We therefore made a detailed comparison of the selectivity profile of H4 and the TbNBT1 activity expressed in yeast (Table 5.1). No significant differences were found between the two sets of data, which are also depicted as a bargraph in Figure 5.5. Though this approach cannot in itself be conclusive, it adds further weight to the hypothesis. Final proof awaits the construction of a genetic knockout strain lacking any copies of TbNBT1.

	NBT1 in Yeast. [*Ki* or *Km*]			H4 in procyclics [*Ki* or *Km*]		
compound	*Avg*	*SE*	*n*	*Avg*	*SE*	*n*
Hypoxanthine[a]	0.66	0.22	4	0.55	0.07	7
Adenine[a]	2.1	0.6	3	2.6	0.4	3
guanine	1.4	0.3	3	2.6	0.6	3
xanthine	4.3	1.1	3	4.5	0.5	3
allopurinol	5.4	1.1	3	2.5	0.4	3
uracil	68.0	6.5	4	94.8	27.4	4
thymine	ND[b]			293.0	62.0	3
cytosine	ND[b]			1251.0	6.0	2
adenosine	1898.4	986.5	3	865.0	177.0	3
inosine	47.4	17.2	4	20.2	7.3	3
guanosine	5.3	1.5	3	4.7	1.4	4
3-Deazaguanine	ND[b]			2.0	0.3	3
7-Deazaguanine	ND[b]			4.9	0.4	3

TABLE 5.1 Kinetic constants of *TbNBT1* expressed in yeast and for the H4 transport activity in procyclic *T. b. brucei*. K_m or K_i values in yeast were determined using either 0.1 μM [^{3}H]adenine or [^{3}H]hypoxanthine as radiolabel for K_i determinations and 25 nM radiolabel to measure K_m values. Experiments with procyclics used 30 or 100 nM [^{3}H]hypoxanthine for the determination of K_m and K_i values, respectively, and an incubation time of 30 s. K_m values were derived from Michaelis-Menten or Eadie-Hofstee plots; K_i values were derived from IC_{50} values as described previously (Wallace, *et al.*, 2002). [a] K_m values. [b] ND, not determined.

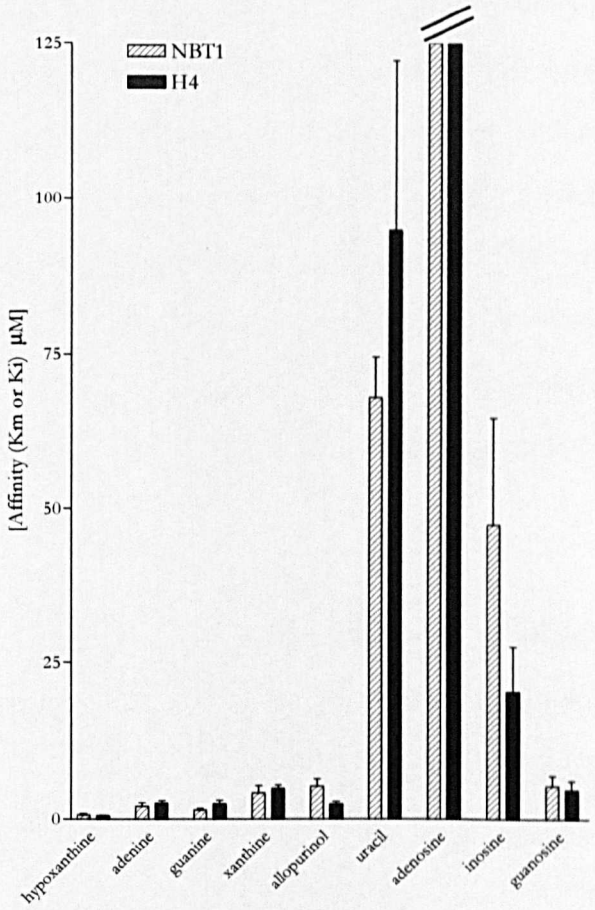


FIGURE 5.5 Kinetic constants of *TbNBT1* expressed in yeast and for the H4 transport activity in procyclic *T. b. brucei*. K_m or K_i values in yeast were determined using either 0.1 µM [^{3}H]adenine or [^{3}H]hypoxanthine as radiolabel for K_i determinations and 25 nM radiolabel to measure K_m values. Experiments with procyclics used 30 or 100 nM [^{3}H]hypoxanthine for the determination of K_m and K_i values, respectively, and an incubation time of 30 s.

5.2.4 Substrate recognition motifs of the *T. b. brucei* H4

The way the *T. b. brucei* H2 transporter binds hypoxanthine has been previously described (Wallace, *et al.*, 2002). Our similar studies described the recognition motifs of the purine nucleobase transporter in *Leishmania major* promastigotes LmajNBT1, the nucleobase transporter of *L. mexicana* amastigotes LmexNBT1 (Al-Salabi and De Koning, 2005) and the human FNT1 nucleobase transporter (Al-Salabi, *et al.*, 2003;

Wallace, *et al.*, 2002). Apart from the obvious pharmacological advantages of understanding what potential antimetabolites may be transported by pathogen and cells, these models provide a functional fingerprint for the transporter, regardless of whether it has been cloned or not. We were thus able to conclude that the LmajNBT1 and TbH2 nucleobase transporters were functional homologues, but bound their substrates entirely different form the corresponding human transporter. It was therefore of interest to see whether the essential architecture of substrate binding would be preserved by a further member of the kinetoplastid nucleobase transporter family.

Certain differences with the H2 binding motif are quickly obvious. Firstly, there was no significant contribution through N3, as the *Ki* values of guanine and 3-deazaguanine were almost identical (Figure 5.2 and Table 5.2). Similarly, the difference in *Ki* value of guanine with 7-deazaguanine was not statistically significant and at most 2-fold, leading to a $\delta(\Delta G^0)$ of just 1.6 kJ/mol. Nor were *Ki* values of adenine and purine, or 6-thioguanine and guanine statistically different, excluding important interactions with position 6 of the purine ring of adenine or hypoxanthine.

However, the difference between 1-deazapurine and purine was highly significant ($P < 0.01$; unpaired *T*-test), leading to an estimated 2.5 kJ/mol for an interaction with the unprotonated N1 of adenine. As the Gibbs free energy of binding of hypoxanthine exceeds that of adenine by 3.9 kJ/mol, the unprotonated N1 hydrogen bond profits only adenine, and the keto group of hypoxanthine does not significantly contribute to binding, the protonated N1(H) must be 6.4 kJ/mol. Furthermore the $\delta(\Delta G^0)$ between guanine and 9-deazaguanine of 3.4 kJ/mol ($P < 0.01$; unpaired *t*-test) indicates a role for N9(H) in binding purines.

Compound	ΔG^0 [kJ/mol]	*n*	*Km* or *Ki*	SE	$\delta[\Delta G^0]$	control
hypoxanthine	35.7	7	0.55	0.07		
adenine	31.9	3	2.63	0.41	-3.88	hypoxanthine
guanine	31.9	3	2.56	0.56	-3.81	hypoxanthine
xanthine	30.3	3	4.98	0.53	-5.46	hypoxanthine
allopurinol	32.0	3	2.48	0.39	-3.73	hypoxanthine
uracil	23.0	4	94.8	27.4	-7.31	xanthine
thymine	20.2	3	293	62	-10.10	xanthine
cytosine	16.6	2	1251	6.00	-19.16	hypoxanthine
adenosine	17.5	3	865.0	177.0	-18.25	hypoxanthine
inosine	26.8	3	20.2	7.3	-8.93	hypoxanthine
guanosine	30.4	4	4.71	1.41	-1.51	guanine
3-deazaguanine	32.6	3	1.98	0.28	0.64	guanine
6-thioguanine	33.6	3	1.31	0.14	1.66	guanine
purine	30.1	3	5.27	1.00	-1.72	adenine
1-deazapurine	27.6	3	14.5	0.36	-2.51	purine
7-deazaguanine	30.3	3	4.90	0.42	-1.61	guanine
9-deazaguanine	28.5	3	10.0	1.1	-3.38	guanine
4-(3H)-pyrimidone	23.0	3	95.3	15.7	-12.78	hypoxanthine
5,6-dihydrouracil	10.9	3	12428	2833	-12.09	uracil
1-deaza5,6dihydrouracil		2	>10 000			
2-aminopurine	31.7	3	2.81	0.70	1.55	purine
06-methylguanine	33.2	3	1.53	0.48	1.52	2-aminopurine

TABLE 5.2 Gibbs free energies (kJ/mol) of substrate interacting with *Trypanosoma brucei brucei* procyclic H4. Gibbs free energy of substrate-transporter interactions was calculated from the K_m and K_i values, using the Nernst equation as described in Materials and Methods. The difference with a control compound, either hypoxanthine as the highest affinity compound or guanine, yielded the $\delta(\Delta G^0)$, the loss in binding energy relative to the control compound.

The interactions at N1 and N9, and a possible weak contribution at N7, however, do not provide the total hypoxanthine binding energy of 35.7 kJ/mol calculated from the K_m value. It must therefore be surmised that the balance is the result of π-stacking, a common phenomenon for purines, which are completely flat and have a completely

conjugated π-electron system. This is particularly hard to investigate using the technique employed here, but one indication is given by the $\delta(\Delta G^0)$ of 12.8 kJ/mol between hypoxanthine and 4-(3H)-pyrimidone. The latter molecule lacks the imidazole part of hypoxanthine and thus any contributions from H-bonds at N9(H) and N7, totalling at most 5 kJ/mol. The remaining 7.8 kJ/mol could only be the contribution of the imidazole ring to π-stacking. It is likely that at least two aromatic amino acids in the H4 binding pocket contribute to the binding of hypoxanthine.

In conclusion, it appears that H4 binds it substrate significantly different from H2, employing π-stacking, rather than H-bonding, more widely. In particular, the H-bond with N3 is absent in H4 and can be considered a distinguishing mark of this transporter, among other protozoan nucleobase transporters analysed to date.

5.3 Discussion

Work from our group recently showed that the ENT-family gene TbNBT1, when expressed in yeast, encodes a nucleobase transporter, with highest affinity for hypoxanthine and adenine, which are transported efficiently (Burchmore, *et al.*, 2003). High affinity was also displayed for the other naturally occurring purine nucleobases, guanine and xanthine. Interestingly, the kinetic profile, displaying a K_m of 0.66 ± 0.22 μM for hypoxanthine did not match any of the known nucleobase transporters of *T. b. brucei*. This chapter describes the identification of a novel nucleobase transport activity in procyclic trypanosomes, and we present evidence that this is the activity encoded by TbNBT1.

The only other potential nucleobase transporter from a protozoan that had been cloned prior to TbNBT1 is the *T. b. brucei* P2 aminopurine transporter encoded by *TbAT1* (Mäser, *et al.*, 1999), which has a similar affinity for adenine and adenosine (Carter and

Fairlamb, 1993; De Koning and Jarvis, 1999). On the basis of its very similar V_{max}/K_m ratio for adenine and adenosine, this transporter must be classified as an aminopurine transporter, rather than a nucleoside or nucleobase transporter. Since our study was published, however, an additional nucleobase transporter gene from *T. b. brucei* and one from *Leishmania major* have been reported (Henriques, *et al.*, 2003; Sanchez, *et al.*, 2004b). The significance of the current study, therefore, lies more in the detailed characterisation, which allowed a positive identification of the gene function in the parasite. It was previously reported that a high affinity hypoxanthine transporter was upregulated in *T. b. brucei* procyclics under conditions of purine stress (De Koning, *et al.*, 2000b). It seems now highly probable that this transporter was TbNBT1/H4, which is a far more efficient tool for salvaging low levels of purines than H1. Similar mechanisms of upregulation of purine salvage enzymes have been reported for the related *Crithidia* spp. (Hall, *et al.*, 1996; Gero, *et al.*, 1997; Gottlieb, 1985; De Koning, *et al.*, 2000b). The current study is further of significance for its demonstration that even very similar transporter activities, such as H2 and H4, can be told apart using a rational approach towards substrate recognition, utilising a relatively small number of selected purine analogues as markers for particular interactions between binding pocket of the individual transporters and their substrate.

Chapter Six

NOVEL ENT NUCLEOSIDE TRANSPORTERS IN *TRYPANOSOMA BRUCEI*; CLONING OF ADDITIONAL GENES AND COMPARISON OF SUBSTRATE-TRANSPORTER INTERACTIONS

6.1 Summary

Trypanosomes, like all protozoan parasites, are incapable of *de novo* purine synthesis. This difference in purine metabolism between these parasites and their mammalian host makes purine metabolism an excellent potential target for the rational design of antiparasitic chemotherapeutic agents. The transport of purines by the protozoan parasite *Trypanosoma brucei brucei* has become of particular interest as these transporters are the first step in the purine salvage pathways. Purine transporters are essential for importing the purine nucleobases and nucleosides, which are vital nutrients for the parasite to use as purine source. In addition, some of these transporters facilitate the uptake of chemotherapeutic agents including both purine analogues and non-purine drugs. The increasing understanding of the basis of substrate recognition of these transporters can provide a lead to the rational design of a drug.

Several *T. b. brucei* nucleoside transporters have been cloned and found to be part of the ENT gene family (Landfear, *et al.*, 2004; De Koning, *et al.*, 2005). In the last few years, examination of the *T. brucei* database in our laboratory has revealed the existence of several additional ENT-like sequences in the genome. Using the TbAT1 protein sequence to probe the *T. brucei* genome, three ENT family members were identified, named as Adenosine Transporter like-A (AT-A), Adenosine Transporter like-B (AT-B) and Adenosine Transporter like-D (AT-D). The predicted amino acid sequences of these genes were found to share a high identity to TbNT2 (47.8%%, 65.6% and 70.4%, respectively). The cloning of AT-A, AT-B and AT-D was performed in our laboratory by Lynsey Wallace (see chapter seven and eight in Lynsey Wallace's PhD thesis). In addition, a family of five more ENT-like sequences were identified *in silico* in our laboratory, designated as AT-E, AT-F, AT-G, AT-H and AT-I. All of these genes possess the signature sequences of ENT-like genes such as TbAT1, TbNT2-7, TbNBT1 and TbNBT8.1. However, these ENT family members, AT-E-I, have not been cloned or functionally characterized to date. A phylogenetic tree with protozoan and human ENT family transporters shows that AT-G aligned within a subgroup together

with TbAT1 and the related AT-A and AT-E sequences, whereas AT-F and AT-H grouped with the TbNBT1 and NT8.1 nucleobase transporters (Figure 6.1).

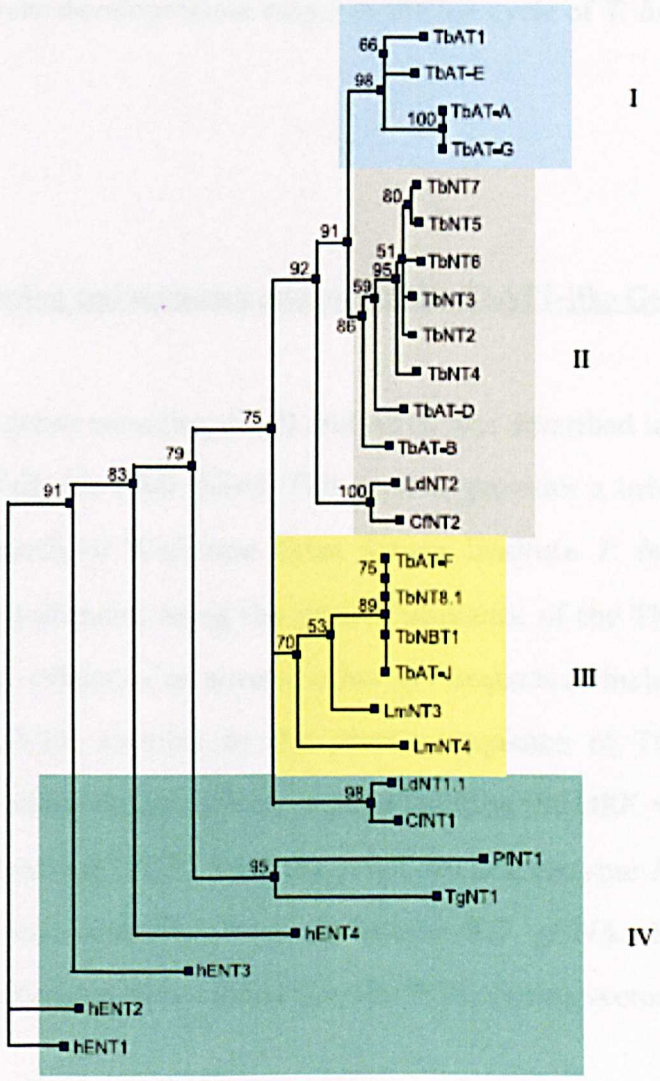


FIGURE 6.1 Phylogenetic relationship of ENT family transporters including protozoan and human members. The tree was aligned using tree-puzzle program and the alignment used was generated by using DIALIGN program (see Appendix II; Aligned sequences of the ENT family transporters showing the identities and the similarities between these members of the family). The lengths of the branches represent the Phylogenetic distance. The tree shown in group *I* is the TbAT1-like genes. The trees shown in groups *II* and *IV* are nucleoside transporter genes. The tree shown in group *III* contains the possible nucleobase transporter genes. Values at the nodes of the tree are bootstrap values based on 10,000 replicates. Reproduced from De Koning, *et al.* (2005).

This chapter describes in great detail the functional characterisation and the substrate selectivity of the Adenosine Transporter like-B (AT-B) and Adenosine Transporter like D (AT-D) expressed in yeast, *Saccharomyces cerevisiae* strain MG887-1, and their expression in different developmental stages in the life cycle of *T. brucei.*

6.2 Results

6.2.1 Molecular cloning and sequence analysis of the TbAT1-like Genes

The cloning of the genes encoding AT-D and AT-B was described in chapter seven and eight in Lynsey Wallace's PhD thesis. This section provides a brief summary of that work. A BLAST search of Wellcome Trust Sanger Institute *T. brucei* sequence data bases by Richard Burchmore, using the protein sequence of the TbAT1 transporter as query, revealed the existence of several other AT-sequences including AT-D and B, with 65.5% and 55.2% identity to the protein sequence of TbAT1, respectively. Oligonucleotide primers, designed from regions flanking the ORF, were employed in a polymerase chain reaction (PCR) using the proof-reading enzyme *Pfu* to amplify DNA fragments of the expected size from *T. brucei* 927 gDNA. Products from two independent PCR reactions were cloned into the PCR cloning vector pGEM-T Easy.

To confirm that all inserts were of the correct predicted size, and possessed the restriction enzyme sites predicted from the sequence retrieved from the genome database, a series of restriction enzyme digests were performed. The cloned products of two independent polymerase chain reactions for AT-D and AT-B were completely sequenced on both strands (MBSU sequencing service, Glasgow, Scotland, UK). In both cases, the two sequences results were found to be identical, and were also identical to those in the genome database. Hydropathy plots for the amino acid sequences of AT-B and AT-D were found to exhibit an apparent secondary structure consistent with a

membrane location and a transport function, and predicted 11 transmembrane regions with the N-terminal on the cytosolic side of the membrane.

The cloned AT-D and AT-B ORFs were excised from the pGEM-T Easy vector by digestion with *NotI* restriction enzyme and subcloned into the *NotI* site of the linearised, de-phosphorylated pDR195 vector. Electrocompetent *S. cerevisiae* cells of strain MG887-1 (*ura3Δ*) were transformed with AT-like: pDR195 constructs using the lithium acetate method. Transformants were selected and cultivated on solid SC medium plates without uracil.

6.2.2 Re-confirmation of insert size and restriction sites

In order to reconfirm the presence of AT-D and AT-B inserts in pDR195 vector before the functional analysis, a series of diagnostic restriction enzyme digests were carried out (Restriction maps shown in Figure 6.2). The products from these digests were analysed on 0.8 % agarose gels in the presence of ethidium bromide (Figure 6.3). Digestion of the AT-D:pDR195 vector construct using *NotI* showed two fragments; the fragment represents the pDR195 vector with a size of ~ 6.3 kb which was the predicted size for this vector. The second fragment obtained represented the released insert AT-D, with a size of ~ 1.4 kb. The control incubation of this construct in the absence of *NotI* led to only one fragment representing the whole construct AT-D:pDR195 (Figure 6.3*A*). In the case of AT-B, digestion resulted in the release of the insert AT-B as a fragment with a size of ~ 1.4 kb, and another fragment for the vector with a size of ~ 6.3 kb (Figure 6.3*B*).

These observations confirmed that both inserts, AT-D and AT-B, were of the correct predicted size, correct orientation, and possessed the restriction enzyme sites predicted from the genome database sequences and our own previous determination of the sequence (L. Wallace's thesis).

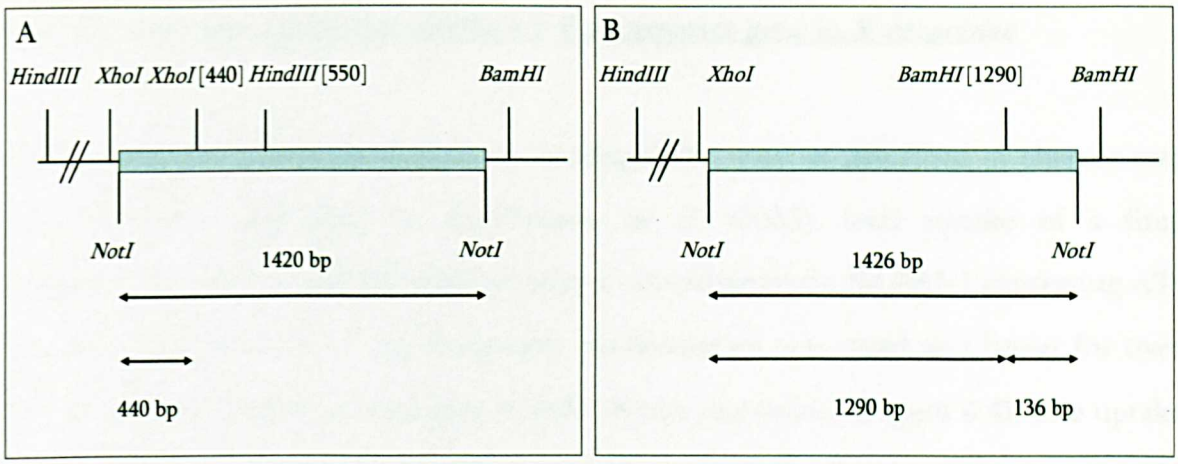


FIGURE 6.2 Restriction maps showing the orientation of AT-B (*A*) and AT-D (*B*) in pDR195 with restriction enzymes. Vector size is 6.3 kb. Reproduced from chapter eight in Lynsey Wallace's PhD thesis.

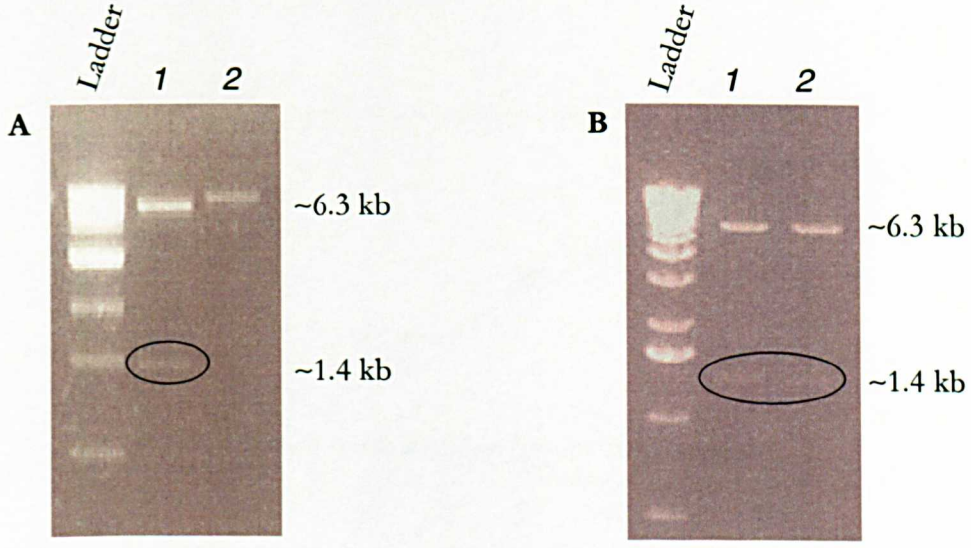


FIGURE 6.3 Agarose gel images confirming the presence of the insert within the vector. A. Digested miniprep of AT-D:pDR195 vector with *NotI* (should release insert from vector), using BioLabs 1 Kb DNA ladder. Digest with *NotI* results in two bands representing the release of AT-D from the vector (*1*) and without *NotI* gives one band for AT-D within the vector (*2*). B. Digestion of duplicate minipreps of AT-B in pDR195, giving identical results: the release of the insert from vector (*1* and *2*). The Invitrogen 1 Kb DNA ladder was used to estimate size.

6.2.3 Kinetic Characterization of the AT-B transporter gene in *S. cerevisiae*

Using a rapid-stop oil-spin technique developed for yeast as described in chapter two and previously described in Burchmore, *et al.* (2003), total uptake of a final concentration of 0.25 μM [^{3}H]adenosine in *S. cerevisiae* strain MG887-1 expressing AT-B within pDR195 vector was examined. Accumulation was rapid and linear for over 120 s with a correlation coefficient of 0.96 (linear regression) (Figure 6.4). The uptake was significantly different from zero with a *P* value of 0.01 (*F* test), with a rate of 0.080 ± 0.011 pmol(10^7 cells)$^{-1}$s^{-1}. The transport activity was saturable in the presence of 1 mM unlabeled adenosine and was not significantly different from zero ($P > 0.4$; *F* test), which confirms that all the adenosine uptake is transporter-mediated. Figure 6.3 Shows the uptake of the 0.25 μM [^{3}H]adenosine in *S. cerevisiae* strain MG887-1 with pDR195 only (control) was not significantly different from zero ($P > 0.2$; *F* test).

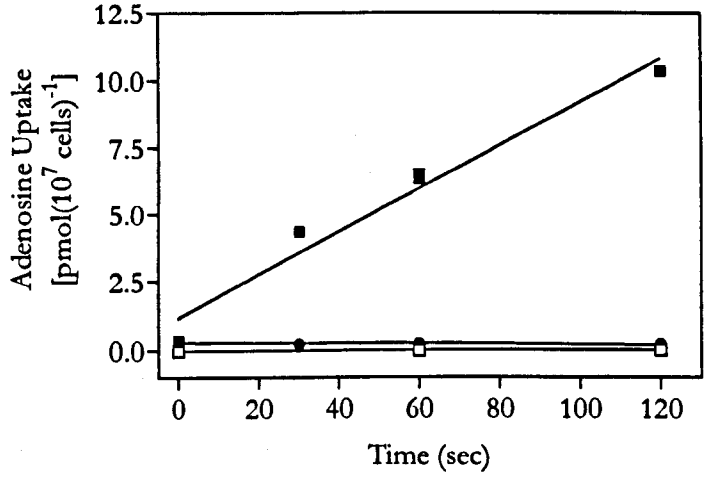


FIGURE 6.4 Transport of [^{3}H]adenosine by AT-B in *S. cerevisiae* strain MG887-1. Uptake of 0.25 μM [^{3}H]adenosine was linear over 120 s (■: $r^2 = 0.96$) as calculated by linear regression. In the presence of 1 mM unlabelled adenosine (□) or the control (empty vector, •), uptake was not significantly different from zero ($P > 0.4$ and $P > 0.2$, respectively; *F* test). In some cases, an apparent positive intercept can be observed for the timecourses in the absence of inhibitor. This is most likely the caused by extracellular binding of radiolabel, which is displaced by the including of 1 mM non-radiolableled substrate.

Further experiments were performed in order to determine the kinetic parameters of this adenosine transporter. The uptake of 0.015 μM [^{3}H]adenosine in *S. cerevisiae* strain MG887-1 expressing AT-B was assessed using an incubation time of 60 s (initial rate of uptake) in the presence of a range of concentrations (0-1000 μM) of unlabeled adenosine. Figure 6.5 (*inset*) shows that the uptake was saturable and complied with simple Michaelis-Menten kinetics, with average K_m and V_{max} values of 0.44 ± 0.02 μM and 0.60 ± 0.05 pmol(10^7 cells)$^{-1}$s^{-1}, respectively (n = 4). This result indicates that the AT-B possess a high affinity for adenosine.

The substrate specificity of the adenosine transporter by AT-B in *S. cerevisiae* was investigated by studying the inhibition effects of purine and pyrimidine nucleosides and nucleobases on adenosine uptake. Figure 6.5 also shows that the purine nucleoside inosine inhibited the uptake of [^{3}H]adenosine with a K_i value of 3.1 ± 0.7 μM (n = 3). Routine inhibition studies of this transporter were subsequently performed at 0.05 μM, as the radioactivity accumulated at 0.015 μM (necessary concentration to determine the K_m) was very low and only just allowed accurate determinations. The purine nucleoside guanosine also inhibited the uptake of 0.050 μM [^{3}H]adenosine with a K_i value of 2.4 ± 0.3 μM (n = 3). The inhibition profiles obtained using the initial rates of the adenosine transporter by AT-B showed that this transporter possess high affinity for purine nucleosides and relatively low affinity for purine nucleobases such as adenine (K_i value of 670 ± 155 μM; n = 4) and hypoxanthine (K_i value of 286 ± 28 μM; n = 3), and no affinity up to 1 mM for pyrimidines such as uridine, thymidine and cytidine.

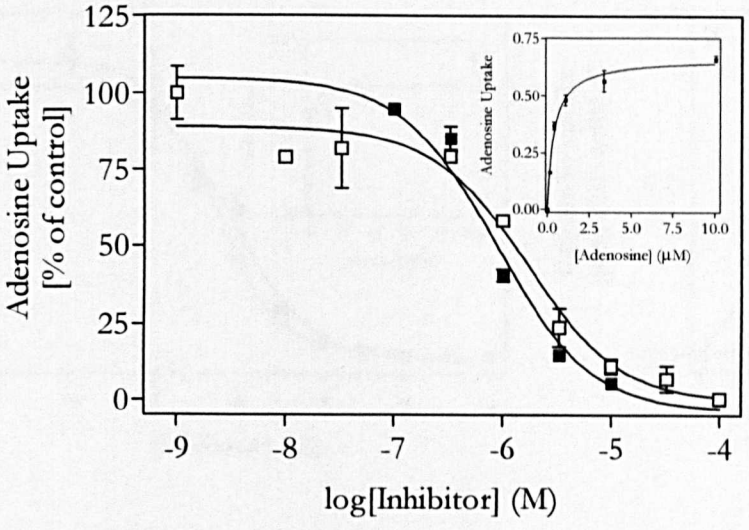


FIGURE 6.5 High affinity adenosine transporter in *S. cerevisiae* strain MG887-1 expressing AT-B. Inhibition of 0.015 μM [^{3}H]adenosine by increasing concentrations of unlabeled adenosine (■; IC_{50} = 0.86 μM) and inosine (□; IC_{50} = 1.6 μM), data were expressed as percentage of control (no inhibitor). Michaelis-Menten plot (*inset*) shows calculated K_m and V_{max} values for this experiment of 0.37 μM and 0.66 pmol(10^7 cells)$^{-1}$s^{-1}, respectively.

To investigate whether separate transporters are involved in the uptake of purine nucleosides such as adenosine, inosine and tubercidin, additional transport studies were performed using [^{3}H]inosine and [^{3}H]tubercidin. The uptake of 50 nM [^{3}H]inosine using incubation time of 120 s (measuring the initial rates of uptake) was inhibited by range of concentration between 0-1000 μM of unlabelled inosine and adenosine (Figure 6.6*A*). The mean *Ki* value for adenosine inhibiting inosine uptake from three separate experiments was 0.48 ± 0.12 μM. Figure 6.6*A* (*inset*) shows the conversion of the inosine inhibition data to a Michaelis-Menten plot of total inosine uptake. The mean values from three separate experiments were 0.53 ± 0.06 μM and 0.25 ± 0.05 pmol(10^7 cells)$^{-1}$s^{-1} for K_m and V_{max}, respectively.

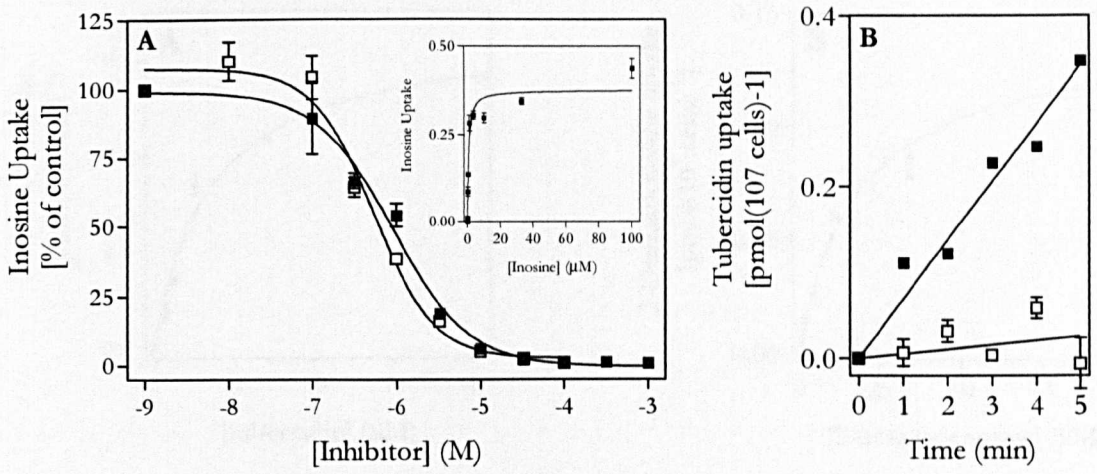


FIGURE 6.6 Transport of labeled purine nucleosides by AT-B in *S. cerevisiae* strain MG887-1. **A.** Transport of 50 nM [^{3}H]inosine over 120 s was inhibited by indicated concentrations of unlabelled inosine (■) or adenosine (□), with IC_{50} values of 0.90 μM and 0.54 μM, respectively. The inset shows the conversion of the inosine inhibition data to a Michaelis-Menten plot of total inosine uptake, with a K_m value of 0.67 μM and a V_{max} of 0.37 pmol(10^7 cells)$^{-1}$s^{-1} for this experiment. **B.** Timecourse of 1 μM [^{3}H]tubercidin uptake in the presence (□) or absence (■) of 1 mM unlabelled adenosine.

Uptake of 1 μM [^{3}H]tubercidin was significantly different from zero with a P value of < 0.0001 (F test), with a rate of 0.068 ± 0.003 pmol(10^7 cells)$^{-1}$s^{-1} (Figure 6.6*B*). However, in the presence of 1 mM unlabeled adenosine transport was not significantly different from zero ($P > 0.1$; F test). The K_m for [^{3}H]tubercidin was determined at 15.1 ± 1.5 μM with a V_{max} of 0.25 ± 0.06 pmol(10^7 cells)$^{-1}$s^{-1} ($n = 3$)(Figure 6.7*A*). Similarly, the K_m for [^{3}H]-3-deazaadenosine was found to be 25.7 ± 4.0 μM with a V_{max} of 0.16 ± 0.04 pmol(10^7 cells)$^{-1}$s^{-1} ($n = 3$; Figure 6.7*B*). We thus know K_m values for four different permeants of AT-B in the *S. cerevisiae* expression system. Since the V_{max}/K_m gives a measure of the efficiency of transport, it follows that AT-B transports adenosine ~ 2.6-fold more efficiently than inosine. While the V_{max} values are all rather similar, this ratio reveals that the interactions at N7, and in particular at N3, are vital for efficient translocation at this transporter (Table 6.1).

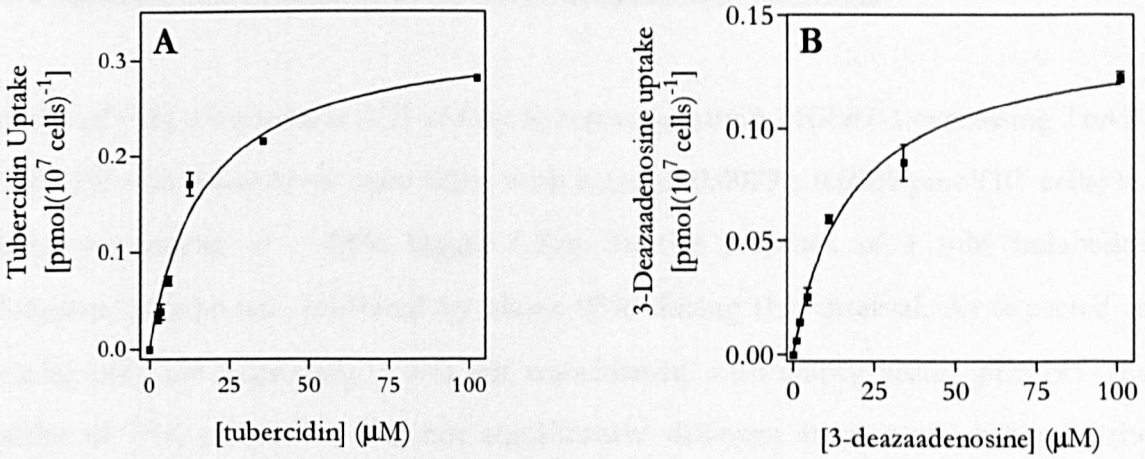


FIGURE 6.7 Purine nucleosides uptake by AT-B in *S. cerevisiae* strain MG887-1. **A.** Shows the conversion of the tubercidin inhibition data to a Michaelis-Menten plot of total tubercidin uptake, with a K_m value of 17.0 μM and a V_{max} of 0.33 pmol(10^7 cells)$^{-1}$s^{-1} for this experiment. **B.** Shows the conversion of the 3-deazaadenosine inhibition data to a Michaelis-Menten plot of total 3-deazaadenosine uptake, with a K_m value of 18.1 μM and a V_{max} of 0.14 pmol(10^7 cells)$^{-1}$s^{-1} for this experiment.

Permeant	K_m	V_{max}	V_{max}/K_m	*n*
Adenosine	0.44 ± 0.02	0.60 ± 0.05	1.4	4
Inosine	0.53 ± 0.06	0.25 ± 0.05	0.47	3
Tubercidin	15 ± 1.5	0.25 ± 0.06	0.02	3
3-deazaadenosine	25 ± 4.0	0.16 ± 0.04	0.006	3

TABLE 6.1 K_m and V_{max} values and their ratios for some purine nucleosides were tested on AT-B in *S. cerevisiae* strain MG887-1. K_m and V_{max} values were expressed in μM and pmol(10^7 cells)$^{-1}$s^{-1}, respectively.

6.2.4 Characterization of the AT-D transporter gene in *S. cerevisiae*

Uptake of [^{3}H] adenosine at 0.25 μM by *S. cerevisiae* strain MG887-1 expressing TbAT-D:pDR195 was linear for at least 120 s, with a rate of 0.0079 ± 0.0005 pmol(10^7 cells)$^{-1}$s^{-1} (linear regression; r^2 = 0.99; Figure 6.8*A*). In the presence of 1 mM unlabelled adenosine, uptake was inhibited by about 95% during this interval. As expected in control cells not expressing AT-D but transformed with empty vector pDR195, the uptake of [^{3}H] adenosine was not significantly different from zero, either in the presence (P = 0.4, F test) or absence (P = 0.9, F test) of 1 mM unlabeled adenosine (Figure 6.8*A*), and the observed positive intercept is most likely caused by extracellular binding of radiolabel, which is displaced by the including of 1 mM non-radiolableled substrate. Figure 6.8*B* confirms the linear adenosine uptake at the much lower concentration of 0.015 μM [^{3}H]adenosine for 240 s (0.0003 ± 0.00003 pmol(10^7 cells)$^{-1}$s^{-1}, linear regression; r^2 = 0.96). The addition of 1 mM unlabeled adenosine and 1 mM unlabeled adenine, inhibited the uptake by > 98% and 65%, respectively (Figure 6.8*B*). This indicates that AT-B has low affinity for adenine, as the transporter was clearly not saturated by 1 mM of the nucleobase.

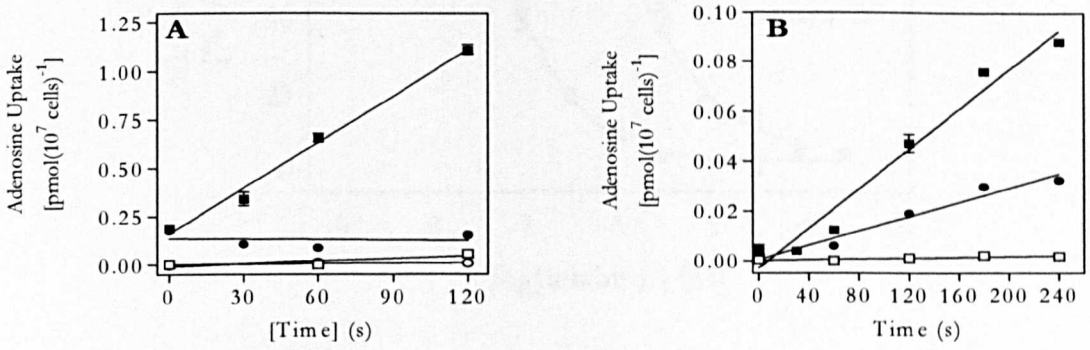


FIGURE 6.8 Linear adenosine transport by AT-D in *S. cerevisiae* strain MG887-1. A. Timecourse of 0.25 μM [^{3}H]adenosine uptake by *S. cerevisiae* transformed with AT-D:pDR195 in the presence (□) or absence (■) of 1 mM unlabelled adenosine or by the empty vector (pDR195) in the absence (●) or presence of 1 mM unlabelled adenosine (○). B. Timecourse of 0.015 μM [^{3}H]adenosine uptake by *S. cerevisiae* strain MG887-1 expressing AT-D in pDR195 in the presence (□) or absence (■) of 1 mM unlabelled adenosine or presence of 1 mM unlabelled adenine (●).

Subsequent experiments with [^{3}H]adenosine were performed using a concentration of 0.05 μM, except for determination of K_m (where 15 nM label was used), and an incubation time of 60 s, within the linear phase of uptake and therefore measuring true initial rates of transport. Figure 6.9 shows that [^{3}H]adenosine transport measured in the presence of various concentrations (0-1000 μM) of unlabeled adenosine was monophasic and conformed to simple Michaelis-Menten kinetics (Figure 6.9, *inset*) and displayed a K_m value of 0.058 ± 0.01 μM (n = 4), which demonstrates that AT-D possesses a very high affinity for adenosine but appears to have a low capacity (V_{max} = 0.011 ± 0.004 pmol(10^7 cells)$^{-1}$s^{-1}; n = 4) when expressed in yeast. To further characterise the substrate specificity of this transporter, inhibition experiments were performed using 50 nM labelled adenosine. The inhibitory effects of potential substrates for AT-D were tested by investigating the effect of varying concentrations of test compounds.

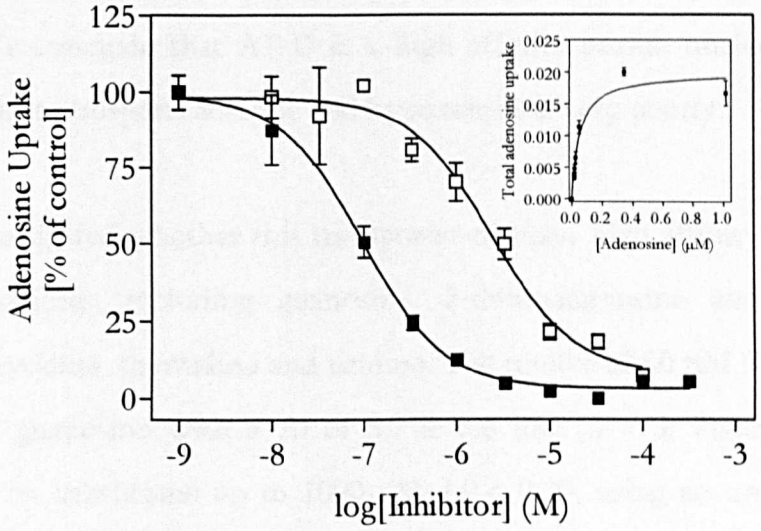


FIGURE 6.9 High affinity adenosine transporter in *S. cerevisiae* strain MG887-1 expressing AT-D. Inhibition of 0.015 μM [^{3}H]adenosine by increasing concentrations of unlabelled unlabelled adenosine (■: IC_{50} = 0.09 μM) or inosine (□; IC_{50} = 2.3 μM), and expressed as percentage of control (no inhibitor). Michaelis-Menten plot (*inset*) shows calculated K_m and V_{max} values for this experiment of 0.046 μM and 0.021 pmol(10^7 cells)$^{-1}$s^{-1}, respectively.

The uptake of [^{3}H] adenosine was inhibited by purine nucleosides. Inosine displayed a *Ki* value of 4.4 ± 1.5 μM (n = 4; Figure 6.9). To assess whether inosine is transported across the plasma membrane by this adenosine transporter, additional transport experiments were performed using labelled inosine. Time course experiments using 0.25 μM [^{3}H] inosine showed that the uptake was linear for at least 5 min (Figure 6.10*A*), with a rate of 0.55 ± 0.02 pmol(10^7 cells)$^{-1}$s^{-1}, and was not significantly different from zero when adding 1 mM of unlabeled inosine (P < 0.28, t test). Figure 6.10*B* shows that the uptake of 50 nM [^{3}H] inosine was saturable when measured using 3 min incubations in the presence of 0 - 1000 μM of unlabeled inosine. The uptake was also inhibited by the same concentration range of unlabeled adenosine (*Ki* = 0.025 ± 0.004 μM). The conversion to simple Michaelis-Menten kinetics resulted in average K_m and V_{max} values of 2.7 ± 0.70 μM and 0.12 ± 0.039 pmol(10^7 cells)$^{-1}$s^{-1}, respectively (Figure 6.10*B*, *inset*). These findings suggest that adenosine and inosine are both substrates of the AT-D transporer, as the K_m value for inosine transport is almost identical to the *Ki* value for inosine when inhibiting [^{3}H]adenosine uptake and vice versa for adenosine transport. We conclude that AT-D is a high affinity purine nucleoside transporter, particularly as it transports adenine and hypoxanthine very poorly.

We next investigated whether this transporter displays high affinity for other natural purine nucleosides, including guanosine, 2-deoxyadenosine and the pyrimidine nucleosides cytidine, thymidine and uridine. The uptake of 50 nM [^{3}H] adenosine was inhibited by guanosine with a *Ki* of 5.7 ± 0.6 μM (n = 3; Figure. 6.11), but not significantly by xanthosine up to 1000 μM, (P < 0.20, using an unpaired student's t test). The inhibition profile of adenosine transporter obtained, showed that AT-D transporter possesses a very high affinity for purine nuceosides and low affinity for purine nucleobases such as adenine (*Ki* = 167 ± 39 μM; n = 3) and hypoxanthine (*Ki* = 299 ± 74 μM; n = 3), as well as all pyrimidine nucleosides tested such as uridine (*Ki* = 219 ± 37 μM; n = 3) (Figure 6.11).

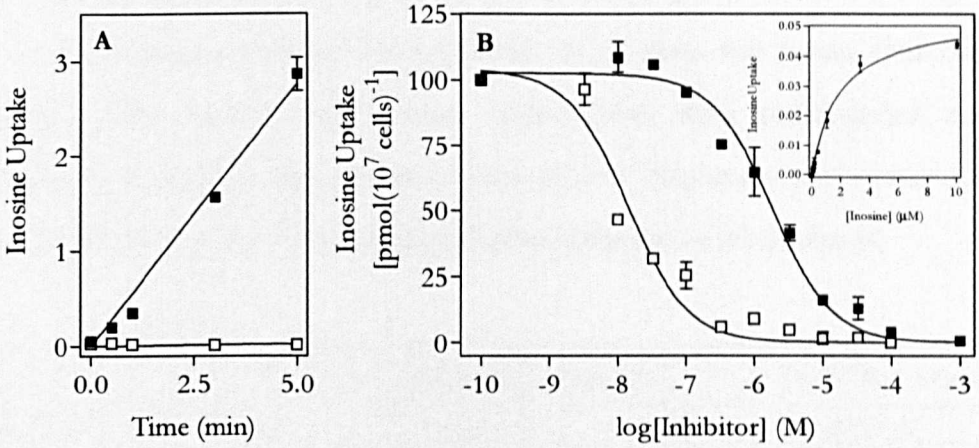


FIGURE 6.10 Inosine transport by AT-D in *S. cerevisiae* strain MG887-1. **A.** Time course for 0.25 μM [^{3}H] inosine uptake in presence (□) or absence (■) of 1 mM of unlabeled inosine. **B.** Inhibition of 0.050 μM [^{3}H]inosine uptake by various concentrations of inosine (■) and adenosine (□) with IC_{50} values for this experiment of 1.8 and 0.014 μM, respectively. Data were expressed as percentage of control, defined as uptake in the absence of inhibitor. The inset shows the conversion of the inosine inhibition plot to a Michaelis-Menten curve, showing total inosine transport with K_m and V_{max} values for this experiment of 1.8 μM and 0.05 pmol(10^7 cells)$^{-1}$s^{-1}, respectively.

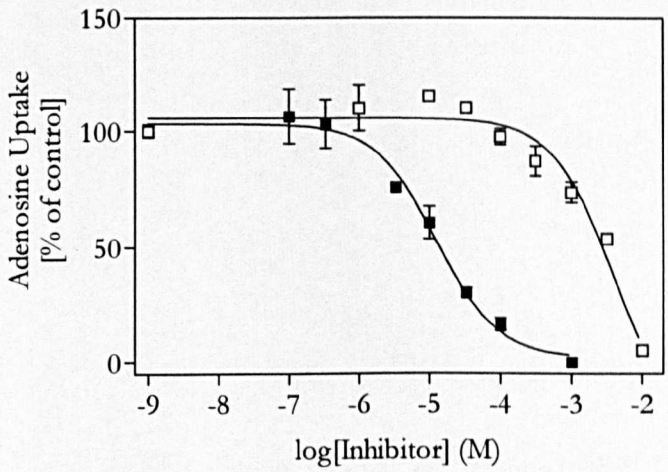


FIGURE 6.11 The effects of purine and pyrimidine nucleosides on the uptake of [^{3}H]adenosine by AT-D in *S. cerevisiae* strain MG887-1. Initial rates of 50 nM [^{3}H]adenosine uptake using incubation time of 60 s, were determined in the presence of various potential inhibitors and expressed as a percentage of control (no inhibitor). Inhibition of 50 nM [^{3}H]adenosine uptake by guanosine (■) and uridine (□) with IC_{50} values of 12.5 μM and 405 μM, respectively (for this experiment).

These values confirmed the specificity of AT-D for purine nucleosides (Table 6.2). Even though, purine nucleosides inhibited AT-D does not mean that they are its permeants. This would need further experiments with radiolabeled nucleosides. Nonetheless, it is clear that the AT-D transporter displays a preference for binding purine nucleosides rather than pyrimidines nucleosides or nucleobases.

	TbAT-D in yeast [μM]		TbAT-B in yeast [μM]	
Compound	*Ki* or K_m	n	*Ki* or K_m	n
Adenosine	0.058 ± 0.01[a]	4	0.41 ± 0.03[a]	5
Inosine	2.7 ± 0.7[a]	3	0.53 ± 0.06[a]	3
Guanosine	6.1 ± 0.6	3	2.4 ± 0.2	3
Xanthosine	>1000	1	NE[1], 1000	4
Uridine	234 ± 39	3	NE[1], 1000	4
Thymidine	505 ± 34	3	NE[1], 1000	3
Cytidine	92 ± 31	3	NE[1], 1000	3
Cordycepin	5.8 ± 1.4	3	31 ± 6.0	4
AraA	1.38 ± 0.2	3	ND[2]	
Tubercidin	2.3 ± 0.2	4	15 ± 1.5[a]	3
6-mercaptopurine riboside	10 ± 0.0	3	29 ± 5	3
2'-deoxyadenosine	0.046 ± 0.006	3	1.0 ± 0.3	4
5'-deoxyadenosine	0.32 ± 0.07	3	0.9 ± 0.1	3
Nebularine	4.0 ± 1.0	3	31 ± 6.7	5
1-deazaadenosine	0.21 ± 0.07	4	1.3 ± 0.4	4
3-deazaadenosine	0.25 ± 0.09	4	25 ± 4.0[a]	3
NA-01 (2-nitroAdo)	88 ± 0.0	1	ND[2]	
NA-63 (2-nitroPurRib)	80 ± 0.0	1	ND[2]	
7-deaza-2'deoxyAdo	4.0 ± 0.6	3	927 ± 38	4
6-chloropurine riboside	12 ± 0.2	2	33 ± 4.4	4
Hypoxanthine	320 ± 80.1	3	284 ± 28	3
Adenine	147 ± 41	3	665 ± 154	4
Guanine	ND[2]		ND[2]	
Xanthine	ND[2]		ND[2]	
Allopurinol	ND[2]		ND[2]	
Uracil	ND[2]		ND[2]	
Thymine	ND[2]		ND[2]	
Cytosine	NE[2], 10000	2	ND[2]	

TABLE 6.2 Kinetic constants of purine nucleoside uptake by AT-D and AT-B transporters in *S. cervisiae*. Kinetic parameters were determined through competitive inhibition of [^{3}H]adenosine or as indicated. In a few cases, extrapolation was required due to limitations of solubility of the inhibitor and based on the assumption of a Hill slope of −1 and eventual 100% inhibition. Extrapolation was not attempted when inhibition at the highest inhibitor concentration was < 50%. Permeant concentrations were 0.25 - 0.015 μM (Routine inhibition studies or determination of the K_m). [a] K_m values. [1] NE, no effect at 1 mM. [2] ND, not determined.

6.2.5 Models for substrate recognition by AT-B and AT-D

The differences in substrate specificity of the P1 and P2 transport activities have been explained in terms of interactions between the transporters and defined functional groups of the substrate (De Koning and Jarvis, 1999) and refined the P1 model slightly to explain its low-affinity binding of uridine (De Koning, *et al.*, 2003). The substrate recognition models for these transporters are displayed in Figure 6.12 and clearly indicate that the main recognition points on the purine ring are N3 and N7 for P1-type binding and N1 and 6(NH_2) for P2, explaining the discrimination of P2, but not of P1, for aminopurines over oxopurines (Carter and Fairlamb, 1993; De Koning and Jarvis, 1999). Estimates of bond energies ΔG^0 (in kJ/mol) have been calculated based on the *Ki* values of competitive inhibitors of [^{3}H]adenosine uptake as described (De Koning and Jarvis, 1999; Wallace, *et al.*, 2002; De Koning, *et al.*, 2005).

However, this method is likely to give an overestimate of bond energies if the translocation rate of the permeant and inhibitor are very different (De Koning, *et al.*, 2005). This has been confirmed for P1, using [^{3}H]tubercidin (7-deazaadenosine) as substrate in *T. b. brucei* bloodstream forms. When used as inhibitor of P1-mediated [^{3}H]adenosine uptake, the *Ki* value was determined as 78 ± 6.4 μM (De Koning and Jarvis, 1999), but the reassessment with [^{3}H]tubercidin determined its K_m as 6.5 ± 1.8 μM (n = 3) (De Koning, *et al.*, unpublished), revaluing the Gibbs free energy of the N7 interaction at 6.8 kJ/mol, down from an estimated 13.4 kJ/mol based on the *Ki* value. Even more important than the more accurate determination of the bond energy, is the determination of V_{max} for tubercidin (0.43 ± 0.10 pmol(10^7 cells)$^{-1}$s^{-1}) and the V_{max}/K_m ratio of 0.077 for P1, showing tubercidin to be > 60-fold less efficient than adenosine as permeant for this transporter, showing that the N7 interaction is more important for efficient translocation than for high affinity. This observation is entirely in agreement with the conclusion of Geiser, *et al* (2005) that tubercidin enters *T. b. brucei* predominantly through the P2 system, which does not significantly interact with N7

(De Koning and Jarvis, 1999). While it is thus much-preferred to use radiolabeled permeant rather than competitive inhibitors to study transporter-substrate interactions, radiolabel is rarely available for any but the most standard nucleosides. To further improve the existing model of interactions between P2 and adenosine, we determined *Ki* values for 1-deazaadenosine (54 ± 16 μM; $n = 5$), nebularine (purine riboside; 17 ± 2 μM; $n = 6$), 6-chloropurine riboside (15 ± 1 μM; $n = 3$) and 9-deazaadenosine (12 ± 3; $n = 3$) using *T. b. brucei* bloodstream forms and 100 nM [^{3}H]adenosine in the presence of 250 μM inosine as described previously (De Koning and Jarvis, 1999), leading to estimates of 7.2 kJ/mol, 10 kJ/mol and 6.4 kJ/mol, respectively, for the P2-interactions with 6-NH2, N1 and N9, respectively, leaving ~10 kJ/mol for the postulated π-stacking to make up the total bonding energy of 34.5 kJ/mol based on the K_m of adenosine (Figure 6.12).

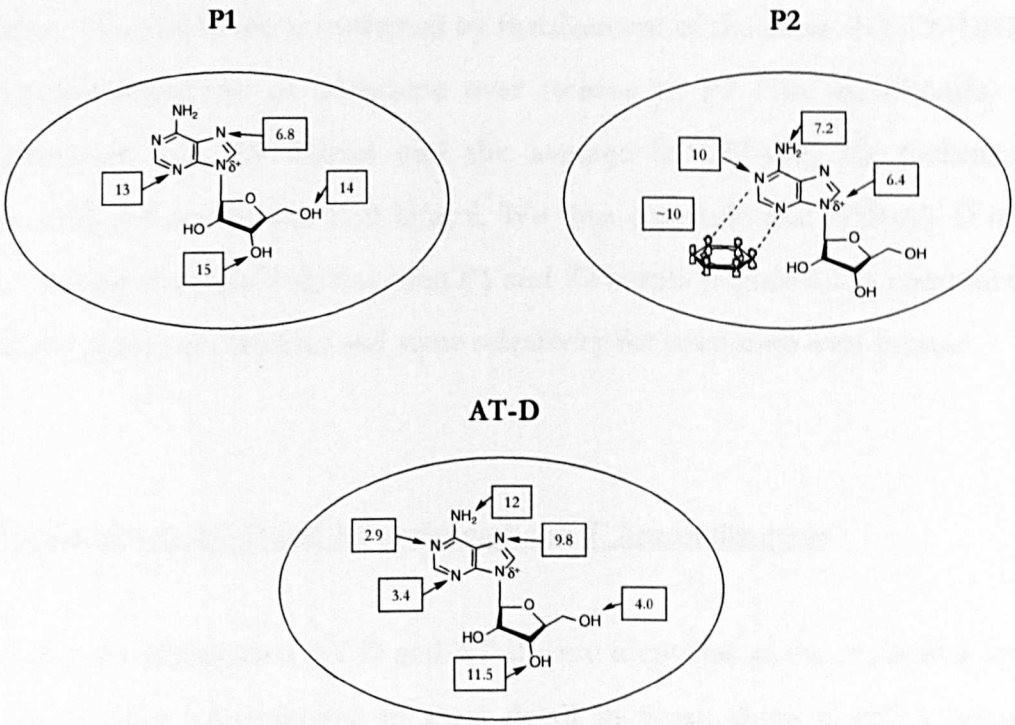


FIGURE 6.12 Model of interactions between adenosine and P1, P2, and AT-D transporters. The AT-D (K_m = 0.058 μM) has much higher affinity than P1 (K_m = 0.38 μM) or P2 (K_m = 0.92 μM), but only for adenosine suggesting that the AT-D binds through P1 motif and main P2 motif. The models for P1 and P2 were adapted from (De Koning and Jarvis, 1999).

We have followed a similar approach to investigate the unusual selectivity of NT9/AT-D. Table 6.3 lists the various analogues tested for inhibition of uptake of 50 nM [^{3}H]adenosine in *S. cerevisiae* transformed with pDR195:AT-D. The preference for purine nucleosides over the corresponding nucleobases is explained by interactions with 3'-OH (Cordycepin (3'-deoxyadenosine) $\delta(\Delta G^0)$ (Ado) = 11.5 kJ/mol) and 5'-OH (5'-deozyadenosine $\delta(\Delta G^0)$ (Ado) = 4.0 kJ/mol) but not 2'-deoxyadenosine ($P > 0.05$, paired *T*-test). The resultant estimate of Gibbs free energy for the ribose group of 15.5 kJ/mol is very similar to the average $\delta(\Delta G^0)$ for adenine and hypoxanthine with their corresponding nucleosides (16.0 kJ/mol). NT9/AT-D also appears to interact with the other contact points of P1-type transporters, N3 and N7. The $\delta(\Delta G^0)$ (Ado) was 3.4 kJ/mol for 3-deazaadenosine, 9.1 kJ/mol for tubercidin and 10.5 for 7-deaza-2'deoxyadenosine. These interactions are sufficient to explain the generally high affinity for purine nucleosides but do not explain the extraordinary high affinity for adenosine. This, however, is explained by involvement of the same -N1=C6-NH2 motif that confers selectivity of adenosine over inosine to P2. The $\delta(\Delta G^0)$(Ado) for 1-deazaadenosine was 2.9 kJ/mol and the average $\delta(\Delta G^0)$(Ado) for 6-chloropurine riboside and nebularine was 12.0 kJ/mol. We thus conclude that NT9-AT-D interacts with adenosine through both the main P1 and P2 motifs (Figure 6.12), combining high affinity for purine nucleosides and some selectivity for adenosine over inosine.

6.2.6 Expression of AT-D and AT-B through the *T. brucei* life cycle

Although both transporters AT-D and AT-B were identified at the molecular level and then functionally characterized in great detail in yeast, there is still a question to answer, which is whether AT-D and AT-B are expressed at all during the *T. brucei* life-cycle and if so, in which developmental stage?

	AT-D in yeast		
Compound	ΔG^0 [kJ/mol]	$\delta[\Delta G^0]$	Control
hypoxanthine	20.7	12.27	Inosine
adenine	22.7	19.81	Adenosine
adenosine	42.5	0.00	Adenosine
inosine	33.0	9.55	Adenosine
guanosine	30.9	2.08	Inosine
uridine	21.5	21.01	Adenosine
thymidine	19.6	22.98	Adenosine
cytidine	23.9	18.60	Adenosine
cordycepin	31.0	11.50	Adenosine
AraA	34.8	7.76	Adenosine
tubercidin	33.4	9.13	Adenosine
2'-deoxyadenosine	43.5	-0.97	Adenosine
5'-deoxyadenosine	38.5	4.03	Adenosine
nebularine	32.0	10.53	Adenosine
1-deazaadenosine	39.7	2.88	Adenosine
3-deazaadenosine	39.2	3.36	Adenosine
NA-01 (2-nitroAdo)	24.1	18.48	Adenosine
NA-63 (2-nitroPurRib)	24.3	18.23	Adenosine
7-deaza-2'deoxyAdo	32.0	10.52	Adenosine
6-chloropurine riboside	29.0	13.52	Adenosine

TABLE 6.3 Gibbs free energies (kJ/mol) of substrate interacting with AT-D in *S. cervisiae*. Gibbs free energy of substrate-transporter interactions was calculated from the K_m and K_i values listed in Table 6.1, using the Nernst equation as described in Chapter two. The difference with a control compound, either adenosine as the highest affinity compound or inosine, yielded the $\delta(\Delta G^0)$, the loss in binding energy relative to the control compound.

African trypanosomes are protozoan parasites that coordinate life cycle differentiation with cell cycle progression (Matthews and Gull, 1994; Mottram, 1994). The trypanosome life cycle is complex, with several distinct stages being present in both the insect vector and mammalian host. During the course of a bloodstream parasitaemia the trypanosomes proliferate initially as morphologically slender forms. As cell numbers increase in the blood, slender forms differentiate to morphologically stumpy forms (Vickerman, 1965).

As described in Chapter two, bloodstream form trypanosomes were grown *in vivo* in mice which were immunocompromised with cyclophosphamide. Parasites were collected at day nine postinfection. At this stage of parasitaemia, pleomorphic lines were predominantly (> 80%) short-stumpy in morphology. Microscopic examination of our preparations showed that stumpy forms were thick and short with an average length of 18 μm and with no free flagellum, but a short one may be present. The stumpy form is characterized by its morphology and through their expression of NADH diaphorase activity (Vickerman, 1965). The NADH diaphorase assay is a reliable cytochemical marker that confirms the identity of slender and stumpy cells. Figure 6.13*A* demonstrates the results from our NADH diaphorase assay as it shows stumpy forms and negative control which was prepared from the same sample but in the absence of the NADH substrate (Figure 6.13*B*). Positive control was included in this experiment using the procyclic form of *T. brucei* (Figure 6.13*C*).

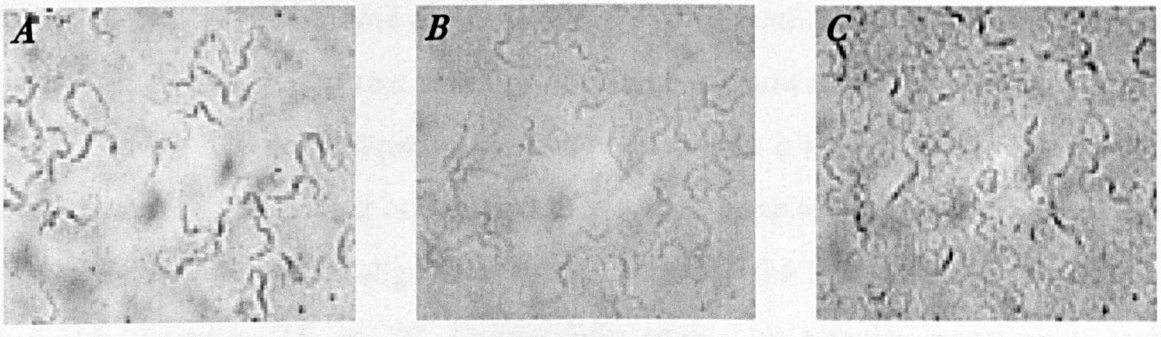


FIGURE 6.13 Representative images of different life stage forms of *T. brucei* taken by immunofluorescence microscopy using the NADH diaphorase assay which was performed to determine the NADH dehydrogenase activity. Short-stumpy bloodstream forms. A. Shows the short-stumpy bloodstream forms incubated for about an hour with the reaction solution. B. Shows the negative control, using the same short-stumpy blood sample, but without adding the NADH substrate. C. Represents the positive control which procyclic forms were used in the same way with the NADH substrate. This assay is to confirm the presence of short-stumpy parasites.

Examination of RNA samples extracted from *T. brucei* of various life cycle stages was carried out to confirm the quality and reliability of the RNA samples used in RT-PCR experiments. As described in Chapter two, concentrations of RNA samples extracted from different strains and life cycle stages of *T. brucei* procyclics of *T. brucei* strain 427, bloodstream forms of *T. brucei* strain 427 in HMI-9 medium, and long-slender or short-stumpy bloodstream forms of *T. brucei* strain 927), were analysed by the Molecular Biology Support Unit (MBSU) of the University of Glasgow. RNA analyses were carried out using either a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies) for quantity or using an Agilent 2100 Bioanalyzer (Agilent Technologies) for quality control. The latter system automatically gives an extremely accurate calculation of the ratio of ribosomal bands in total RNA samples and shows the percentage of ribosomal impurities in mRNA samples. Most importantly, it provides an accurate estimate of the concentration, and the integrity of the RNA is verified. The system plots fluorescence intensity versus migration time and produces electropherograms for each sample (peaks) such as 18S and 28S peaks (Figure 6.14*A*). The data can also be displayed as a densitometry plot, creating a gel-like image (bands), with the help of a ladder that contains fragments of known sizes and concentrations (Figure 6.14*B*). The peak migrating just before 24 seconds is known as the spike control and is found in the loading buffer used to run each sample. The Agilent Bioanalyzer software uses this peak to align each sample to that of the ladder. The peak height is typically between 15 to 20 fluorescence units. In samples containing high concentrations this peak may go unnoticed due to the scaling of the graph. The low baseline, and the sharp 18S and 28S ribosomal peaks indicate that the total RNA is of good quality. Comparing our results with previous RNA analyses using northern hybridisation (Figure 6.14*C*)., showed that the RNA samples used in our experiments were very reliable, as tested for quality and quantity using highly accurate methods.

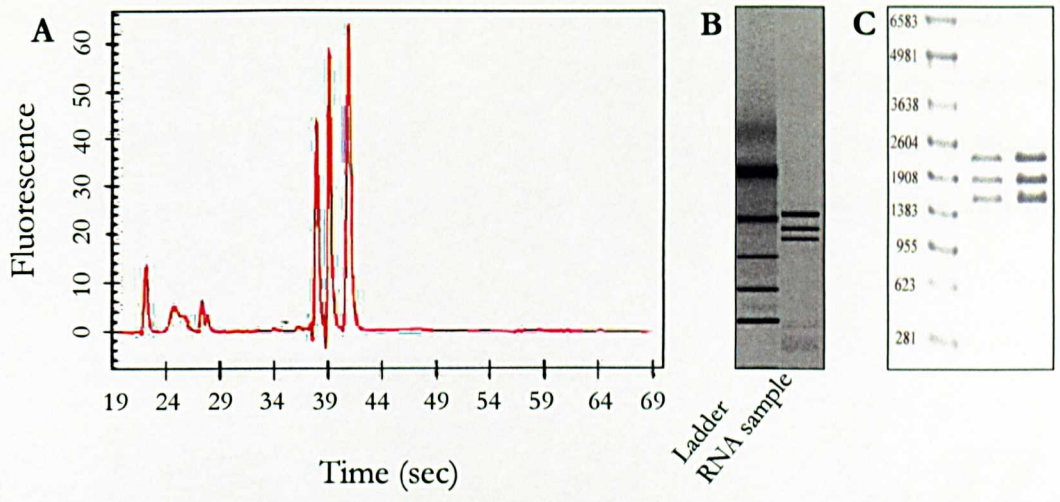


FIGURE 6.14 Examination of RNA samples. The output from the Agilent 2100 Bioanalyzer (results displayed as an electropherogram) showing the control peak and 18S, 28S ribosomal peaks (*A*), and as a gel-like image shown RNA bands with ladder that contains fragments of known sizes (*B*). Transfer of RNA to membrane for Nothern blot, obtained from Chapter eight in Lynsey Wallace's PhD thesis (*C*).

As described in Chapter two, cDNAs were generated from RNA samples extracted from different strains and life cycle stages of *T. brucei* (procyclics of *T. brucei* strain 427, bloodstream forms of *T. brucei* strain 427 in HMI-9 medium, and long-slender or short-stumpy bloodstream forms of *T. brucei* strain 927) by using a SuperScript™ II Reverse Transcriptase (RT). cDNAs were then used as templates in polymerase chain reactions with specific PCR primers for AT-B, AT-D, and controls (TbAT1 and TbNBT1). Details of the primers used for amplification of the AT-B, AT-D, and controls are listed in Appendix II. Negative controls were also included in our PCR experiments (from first-strand reaction) (see Chapter two). PCR products were analysed by electrophoresis in agarose gel (usually 0.8% or 1.2%) containing 0.3 μg/ml of ethidium bromide, using Invitrogen 1 Kb DNA ladder or BioLabs 1 Kb DNA ladder. The results obtained from agarose gels showed the amplification of AT-B fragment from short-stumpy bloodstream form of *T. brucei* 927 with the expected size of ~ 1.4 kb (Figure 6.15*A*). The AT-D fragment was amplified from procyclic form of *T. brucei* 427 with the expected size of ~ 1.4 kb (Figure 6.15*B*). However, no bands were detected for AT-

B or AT-D with long-slender bloodstream forms (Figure 6.15*C*), confirming that the 10-20 % of this life cycle stage cells effect on the result from the 80 % of short-stumpy forms. The same fragment of TbNBT1 (positive control) was amplified in both stages of the life cycle with a size of of ~ 0.7 kb. The Invitrogen 1 Kb DNA ladder was used to estimate sizes in *A* and *B*.

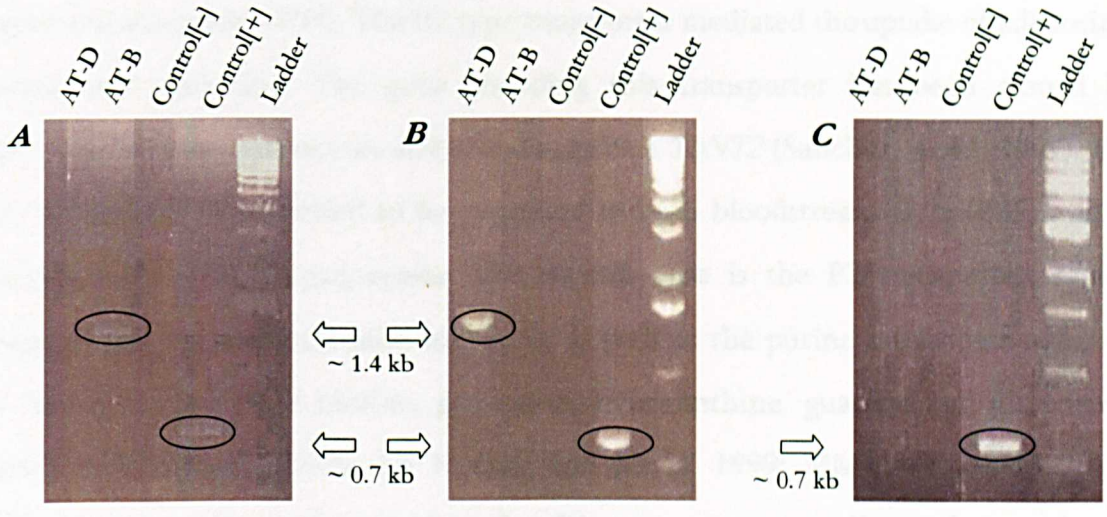


FIGURE 6.15 Agarose gel images confirming the detection of AT-B and AT-D using specific PCR primers listed in appendix II. **A.** Amplification of AT-B fragment from short-stumpy bloodstream form of *T. brucei* 927 with the expected size of ~ 1.4 kb. **B.** Amplification of AT-D fragment from procyclic form of *T. brucei* 427 with the expected size of ~ 1.4 kb. **C.** Represents the gel using long-slender bloodstream forms, showing no bands for AT-B or AT-D. The same fragment of TbNBT1 (positive control) was amplified in all stages of the life cycle with a size of of ~ 0.7 kb The Invitrogen 1 Kb DNA ladder was used to estimate sizes in our experiments.

6.3 Discussion

While an increasing number of nucleoside transporters have been identified and biochemically characterized in recent years, until recently very little was known about protozoan purine transporters at the molecular level, including their cloning and functionally expression. In 1993, two different types of nucleoside transporter were identified in the protozoan parasite *Trypanosoma brucei* and designated P1 and P2 (Carter and Fairlamb, 1993). The P1 type transporter mediated the uptake of adenosine, inosine and guanosine. The gene encoding this transporter has been cloned by expression in *Xenopus* oocytes and was designated *TbNT2* (Sanchez, *et al.,* 1999). P1-type transporters were found to be expressed in both bloodstream form (BSF) and in procyclic form (PF) trypanosomes. The second type is the P2 transporter, which transports the purine nucleoside adenosine as well as the purine nucleobase adenine, but has low affinity for inosine, guanosine, hypoxanthine, guanine and allopurinol (Carter and Fairlamb, 1993; De Koning and Jarvis, 1999; Mäser, *et al.,* 1999). In addition, it was demonstrated that the P2 type system mediates the uptake of antitrypanosomiasis drugs such as pentamidine, melarsoprol and diminazene aceturate (Carter, *et al.,* 1995; De Koning, *et al.,* 2000a; De Koning and Jarvis, 2001). *TbAT1*, the gene encoding the P2 transporter, was the first *T. b. brucei* nucleoside transporter gene cloned (by functional complementation in *Saccharomyces cerevisiae* naturally deficient in purine nucleoside uptake (Mäser, *et al.,* 1999)), and this transporter is expressed only in the long-slender bloodstream stage of the life cycle. Sequence alignments, supported by membrane topology predictions, reveal that all of the protozoan nucleoside and nucleobase transporters identified to date belong to the ENT nucleoside transporter family (Landfear, *et al.*, 2004; De Koning, *et al.*, 2005) exemplified by the human equilibrative nucleoside transporter hENT1 (Griffiths, *et al*, 1997b).

A cluster of six genes has been identified on chromosome 2 of *T. brucei* that apparently encodes ENT-family transporters, designated as TbNT2, TbNT3, TbNT4, TbNT5,

TbNT6, and TbNT7 (Sanchez, *et al.*, 2002). The predicted amino acid sequences of these genes were found to have a high identity to each other. When expressed in *Xenopus laevis* oocytes, the TbNT2, TbNT5, TbNT6, and TbNT7 showed high affinity for adenosine and inosine. However, the TbNT5, TbNT6 and TbNT7 also appeared to be capable of hypoxanthine transport, though with relatively low affinity (Sanchez, *et al.*, 2002). The group of Landfear has identified further members from the ENT family by searching of *T. brucei* database, named as TbNT8, TbNT9, TbNT10 and TbNT11 (Landfear, *et al.*, 2004). It has been suggested that the TbNT8 represents a family of clustered genes (Landfear, *et al.*, 2004). The TbNT8.1 is one member of this family and it was found to be a nucleobase transporter (Henriques, *et al.*, 2003) very similar to TbNTB1 identified and characterized in our laboratory (Burchmore, *et al.*, 2003). Both TbNT8.1 and TbNBT1 appeared to be expressed in procyclic and in bloodstream forms (Burchmore, *et al.*, 2003; Henriques, *et al.*, 2003). The *TbNT10* has been expressed in *Saccharomyces cerevisiae*, and shown to encode a high-affinity nucleoside transporter. Its mRNA has been reported to be expressed in the nondividing short stumpy stage (Sanchez, *et al.*, 2004a). It has also been reported that TbNT11 transporter are represented by two closely related genes, TbNT11.1 and TbNT11.2, but they have not been functionally characterised yet (Landfear, *et al.*, 2004).

Our own laboratories, meanwhile, cloned a number of *T. b. brucei* genes provisionally designated AT-A, AT-B, AT-C, AT-D until a more functional designation could be proposed. AT-C was a nucleobase transporter, subsequently named TbNBT1 (Burchmore, *et al.*, 2003), and AT-A appears not to transport any nucleosides or nucleobases but preliminary results indicate it may be a pentamidine transporter (De Koning, unpublished). The current chapter has dealt with AT-B and AT-D, which were called NT10 and NT9, respectively, by the Landfear group (Landfear, *et al.*, 2004). Both genes were expressed in our *S. cerevisiae* expression system, that was also used for the cloning and characterisation of TbNBT1 (Burchmore, *et al.*, 2003) and were shown to

be broad-specificity purine nucleoside transporters of the P1-type. Phylogenetic analysis also clustered these genes in that category (De Koning, *et al.*, 2005).

A limited characterisation of the TbNT10/AT-B gene has been reported by Sanchez, *et al.* (2004), showing adenosine, inosine and guanosine to be permeants with similar K_m values (~ 0.5 – 1.0 μM). In contrast, this transporter did not mediate uptake of hypoxanthine or other purine nucleobases in the yeast expression system used (Sanchez, *et al.*, 2004a). Our own observations, summarized in Table 6.2, are entirely in agreement with these earlier observations and confirm NT10/AT-B as a P1-type transporter expressed specifically in short-stumpy bloodstream forms.

The same group has also mentioned in a recent review that NT9/AT-D, expressed in *Xenopus* oocytes, is recognised by adenosine, inosine, guanosine and hypoxanthine (Landfear, *et al.*, 2004), but no details or data were provided. We here report a full characterisation of this unusual transporter. As other P1-type transporters, it is predominantly a purine nucleoside transporter, with at best a secondary capacity to transport nucleobases or pyrimidines. But NT9/AT-D displayed much higher affinity for adenosine than any other nucleoside transporter yet reported, with a K_m of 58 ± 13 nM. Unique among the *T. b. brucei* nucleoside transporters, showed > 40-fold higher affinity for adenosine over inosine, whereas all other P1-type transporters have failed to discriminate to any large extend (Carter and Fairlamb, 1993; Sanchez, *et al.*, 1999; Sanchez, *et al.*, 2002; Sanchez, *et al.*, 2004a; De Koning, *et al.*, 2005). This much-increased selectivity seems to be the result of interactions at the $-N_1=C_6-NH_2$ amidine-like motif of adenosine, which has hitherto been associated with recognition by the P2-transport activity rather than P1-type transporters (De Koning and Jarvis, 1999; Barrett and Fairlamb, 1999; De Koning, *et al.*, 2005). Yet, NT9/AT-D still binds inosine with high affinity, apparently utilising all the interactions previously determined for P1: N3, N7, 3'-OH and 5'-OH (De Koning and Jarvis, 1999). It therefore follows that the extraordinarily high affinity of NT9/AT-D is the result of adenosine binding through

both the main P1 and P2 motifs. Since the P2 motif does not contribute to inosine binding, this transporter is most efficient at transporting adenosine, as judged by its V_{max}/K_m. Its expression in procyclics may reflect the low purine concentrations procyclic trypanosomes will experience in their environment, the tsetse midgut. It is known that procyclic *T. b. brucei* up-regulate a high-affinity hypoxanthine transporter during purine starvation (De Koning, *et al*, 2000b).

Chapter Seven

IDENTIFICATION OF HIGH AFFINITY PURINE TRANSPORTERS IN THE APICOPLEXAN PARASITE *TOXOPLASMA GONDII*

7.1 Summary

In the previous Chapters, we have identified the nucleobase transporters in parasites of Trypanosomatidae protozoa such as *Leishmania major* promastigotes (Chapter three) and *Leishmania mexicana* amastigotes (Chapter four), and *Trypanosoma brucei brucei* (Chapters five and six), and characterized them in great detail. We have developed a quantitative model for substrate binding by these transporters and postulated the concept of functional conservation (as opposed to genetic conservation). This stage of my project aims to further our understanding of the salvage of nutrients by the intracellular Apicomplexan protozoan *Toxoplasma gondii* and compare its salvage mechanisms with those of extracellular parasites such as *T. brucei*. Specifically, transport proteins for nucleobases and nucleosides have been identified and characterized with respect to substrate recognition and kinetic parameters.

Purine salvage pathways in the Apicomplexan parasites *Toxoplasma gondii* have been previously studied in great detail and these reports have shown that *T. gondii*, like other members of protozoan parasites, cannot synthesize purines *de novo* and must salvage them from the host (Perotto, *et al.*, 1971; Schwartzman and Pfefferkorn, 1982; Berens, *et al.*, 1995). Therefore purine transporters and salvage enzymes are potential targets or conduits for the development of improved anti-*Toxoplasma* drugs (Ullman and Carter, 1995; Baldwin, *et al.*, 1999; Chaudhary, *et al.*, 2004).

T. gondii has two purine salvage pathways. Hypoxanthine and guanine are rapidly taken up by extracellular tachyzoites (Schwartzman and Pfefferkorn, 1982) and incorporated into their nucleotides by a phosphoribosyltransferase (Krug, *et al.*, 1989; Heroux, *et al.*, 2000; White, *et al.*, 2000). The enzyme responsible for phosphoribosylation of 6-oxypurines, hypoxanthine-xanthine-guanine-phosphoribosyltransferase (HXGPRT), has been analysed in detail both as a drug target itself (Wang, 1984; Ullman and Carter, 1995), and also as a tool in genetic manipulation

of *Toxoplasma* (Donald, *et al.*, 1996; Knoll and Boothroyd, 1998; Matrajt, *et al.*, 2002; Striepen, *et al.*, 2002; Chaudhary, *et al.*, 2005). The source of adenosine nucleotides for *T. gondii* is more enigmatic. The parasites lack an uptake system for adenine nucleotides themselves (Ngo, *et al.*, 2000), but it was reported that tachyzoites are capable of using adenine nucleotides as a purine source after degradation to adenosine (Schwartzman and Pfefferkorn, 1982). A nucleoside triphosphate hydrolase (NTPase) capable of hydrolysing ATP to AMP is released by the parasite into the parasitophorous vacuole in which the parasite resides intracellularly (Asai, *et al.*, 1983; Asai, *et al.*, 1995). However, tachyzoites appear to lack an ecto 5'-nucleotidase that could hydrolyse the AMP to adenosine for subsequent uptake and so the route by which nucleotides are used remains to be elucidated (Ngo, *et al.*, 2000).

Adenosine itself can be incorporated by *T. gondii* directly into AMP *via* adenosine kinase and as this parasite enzyme is at a ten-fold higher activity than HXGPRT (Krug, *et al.*, 1989), it has been proposed that adenosine is the preferred source of purines for the parasite (Krug, *et al.*, 1989). Consistent with this theory, an adenosine transporter (TgAT1) was identified in the plasma membrane of *T. gondii* tachyzoites (Schwab, *et al.*, 1995). However TgAT1 has only a low affinity for the nucleoside, which casts doubt on it being the main entry mechanism for adenosine, which is thought to be present only at sub-micromolar concentrations in the host cell cytoplasm and parasitophorous vacuole. Moreover, mutants of *T. gondii* lacking HXGPRT (Donald, *et al.*, 1996), the TgAT1 adenosine transporter (Chiang, *et al.*, 1999), and adenosine kinase (Darling, *et al.*, 1999) have now all been created and, surprisingly, all are viable. Thus there was a need to re-evaluate the mechanism of uptake, and importance as a source of purine, of adenosine. There is very little known about the mechanism of purine uptake, and as this represents the first step of the parasite's purine metabolism, this makes purine nucleobase and nucleoside transporters an attractive target for chemotherapy.

The work with *Toxoplasma gondii* has led to major new insights in nutrient salvage in apicomplexan parasites, with the identification of novel high affinity nucleoside (TgAT2) and nucleobase (TgNBT1) transporters. A detailed model of substrate binding by TgAT2, explaining its unique high affinity for all physiological nucleosides, has been created. This work was performed in collaboration with other research groups within the Division of Infection and Immunity, and has, in part, been previously published as De Koning et al. (2003) Int J Parasitol. 33:821-31 (see Appendix III).

7.2 Results

7.2.1 Purine nucleobase is taken up by a high affinity hypoxanthine transporter

Figure 7.1*A* shows that uptake of [^{3}H]hypoxanthine at 0.1 μM, 1 μM and 10 μM by *T. gondii* tachyzoites, was rapid and linear for up to 180 s, with rates of 1.0 ± 0.3 x 10^{-4}, 4.3 ± 0.2 x 10^{-4} and 1.75 ± 0.09 x 10^{-3} pmol(10^7 parasites)$^{-1}$s^{-1}, respectively (calculated by linear regression). [^{3}H]Hypoxanthine transport at concentration of 0.3 μM measured at 120 s over a range of 0 - 1000 μM of unlabeled hypoxanthine was saturable and complied with simple Michaelis-Menten kinetics, with a K_m value of 0.91 ± 0.19 μM and a V_{max} value of 0.0045 ± 0.0014 pmol (10^7 parasites)$^{-1}$s^{-1} (n = 3) (Figure 7.1*B* and *inset*).

The inhibitory effects of potential substrates for this hypoxanthine transporter, designated TgNBT1, were tested by measuring initial uptake rates of 0.3 μM [^{3}H]hypoxanthine in the presence of varying concentrations of test compounds (Figure 7.2). The 6-keto nucleobases, guanine (6.9 μM) and xanthine (134 μM) inhibited hypoxanthine transport by 50.2 ± 2.9 % (P < 0.005) and 61.4 ± 2.1 % (P < 0.005), respectively, whereas the aminopurine adenine and the nucleosides, adenosine and inosine, were less potent inhibitors (21.0 ± 3.8 %, 50.5 ± 2.0 % and 47.6 ± 5.7 %,

respectively, at 1 mM). The hypoxanthine analogue allopurinol also weakly inhibited hypoxanthine transport, by 29.7 ± 8.4 % at 1 mM ($P < 0.05$). Whereas, pyrimidines, such as uracil, had almost no effect up to 1mM on hypoxanthine transporter (Figure 7.2).

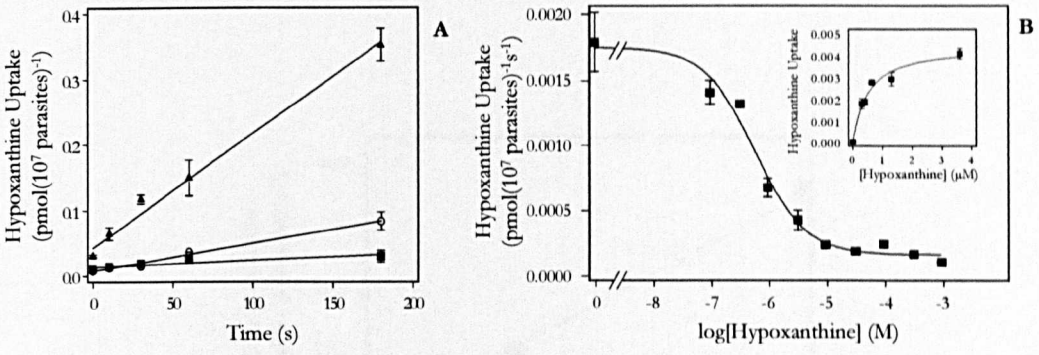


FIGURE 7.1 Nucleobase uptake in *T. gondii* tachyzoites. A. Uptake of 100 nM (■), 1.0 μM (○) or 10 μM (▲) [^{3}H]hypoxanthine was linear for 180 s. B. Inhibition of 0.1 μM [^{3}H]hypoxanthine uptake by 0-1000 μM unlabeled hypoxanthine (IC_{50} = 0.59 ± 0.12 μM). Michaelis-Menten plot (inset) shows calculated K_m and V_{max} values for this experiment of 0.49 μM and 45 x 10^{-4} pmol(10^7 parasites)$^{-1}$s^{-1}, respectively.

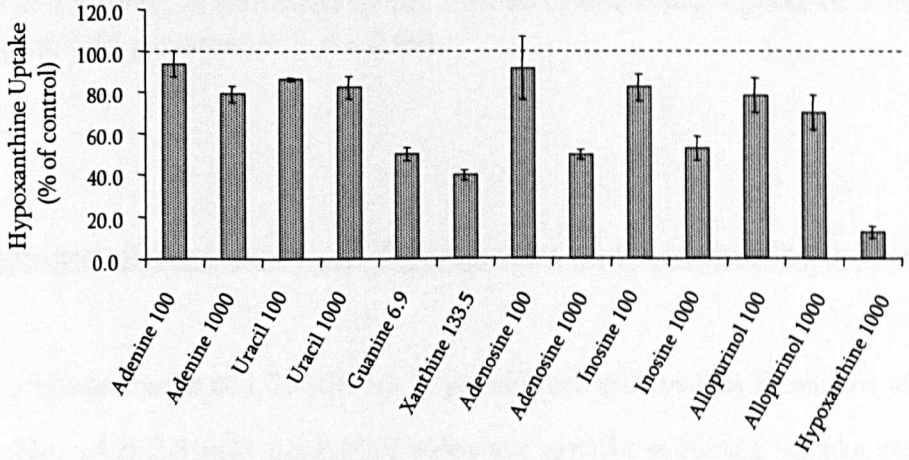


FIGURE 7.2 Inhibition profile of the *T. gondii* nucleobase transporter, TgNBT1. Uptake of [^{3}H]hypoxanthine (0.3 μM) was measured in the presence of various inhibitors and expressed as percentage of control, defined as [^{3}H]hypoxanthine uptake in the absence of inhibitor. Guanine (6.9 μM; $P < 0.005$), xanthine (133.5 μM; $P < 0.005$), adenosine (1 mM; $P < 0.005$) and inosine (1 mM; $P < 0.05$) significantly reduced the transport of hypoxanthine. Data shown are the average of three independent experiments and S.E.

Uptake of [^{3}H]adenine at 0.1 μM, 1 μM and 10 μM by extracellular *T. gondii* tachyzoites was comparatively slow and linear for up to 180 s, with rates of 8.2 ± 2.0 x 10^{-6} pmol(10^7 parasites)$^{-1}$s^{-1}, 6.8 ± 0.89 x 10^{-5} pmol(10^7 parasites)$^{-1}$s^{-1} and 3.9 ± 0.7 x 10^{-4} pmol(10^7 parasites)$^{-1}$s^{-1}, respectively. At 1 μM [^{3}H]adenine, uptake was not saturable by unlabeled adenine concentrations up to 1 mM (Figure 7.3).

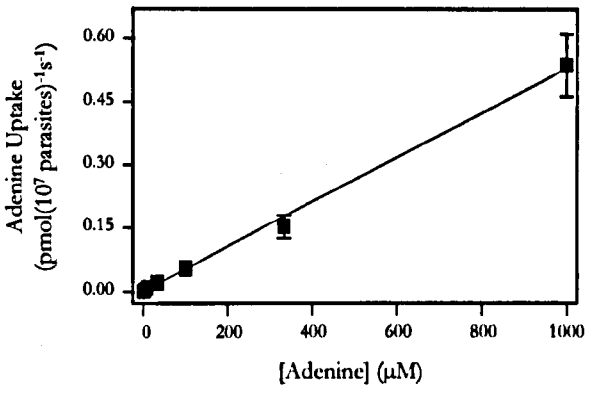


FIGURE 7.3 Uptake of 1 μM [^{3}H]adenine in *T. gondii* tachyzoites was determined at 120 s in the presence of unlabeled permeant (1-1000 μM). No carrier-mediated transport of adenine was observed, as indicated by the non-saturable linear uptake of adenine (5.3 ± 0.1 x 10^{-4} pmol(10^7 parasites)$^{-1}$s^{-1}; r^2 = 0.99).

7.2.2 Tachyzoites of *Toxoplasma gondii* possess two adenosine/inosine transporters

Uptake of [^{3}H]adenosine at 1.25 μM by *T. gondii* tachyzoites was linear for at least 90 s, and saturable, with 2.5 mM unlabeled substrate greatly reducing uptake rates (Figure 7.4*A*). The residual rate in the presence of 2.5 mM unlabeled substrate was assumed to be due to diffusion and in subsequent experiments uptake with 2.5 mM unlabeled adenosine was subtracted from each data point to calculate the mediated uptake. [^{3}H]Adenosine transport (1.25 μM) in the presence of unlabelled substrate (0 - 1000 μM) conformed to simple Michaelis-Menten kinetics (Figure 7.4*B, inset*) with K_m and

V_{max} values of 105 ± 22 μM (n = 3) and 0.066 ± 0.002 pmol(10^7 parasites)$^{-1}s^{-1}$ (n = 3), respectively.

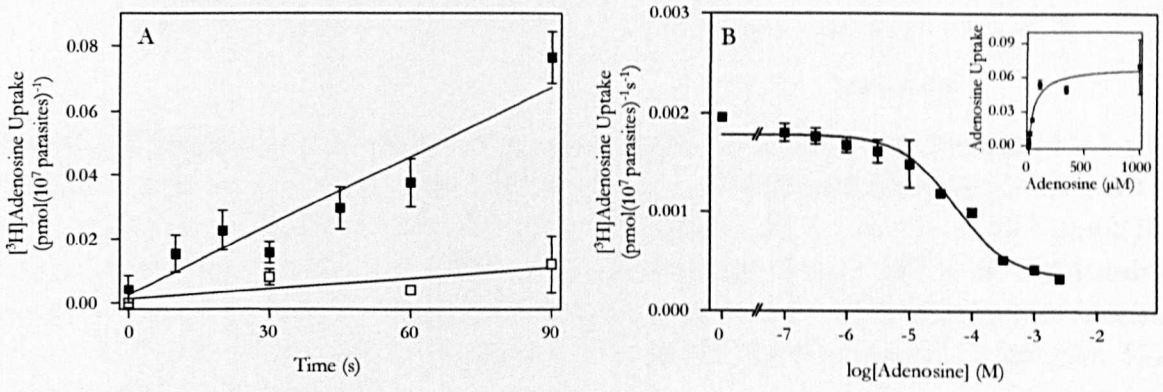


FIGURE 7.4 Transport of [³H]adenosine in extracellular tachyzoites. **A.** Uptake of 1.25 μM [³H]adenosine was measured over various intervals between 0 and 90 s in the presence (□; rate = 1.1 ± 0.6 x 10^{-4} pmol/10^7 parasites/s) or absence (■; rate = 7.2 ± 1.0 x 10^{-4} pmol/10^7 parasites/s) of 2.5 mM unlabeled adenosine. **B.** Uptake of 1.25 μM [³H]adenosine (measured over 45 s) in the presence of various concentrations of unlabeled adenosine (IC_{50} = 64 ± 17 μM). Inset shows the conversion to a Michaelis-Menten plot, yielding calculated K_m and V_{max} values, for this experiment, of 55 ± 14 μM and 0.069 ± 0.004 pmol(10^7 parasites)$^{-1}s^{-1}$, respectively.

Transport of [³H]inosine at 0.1 μM, 1 μM and 10 μM was linear for up to 180 s (Figure 7.5*A*). Uptake of [³H]inosine (1 μM), measured at 120 s, was saturable by unlabelled inosine over the range 0 - 10 mM and exhibited Michaelis-Menten kinetics (Figure 7.5*B* and *inset*). K_m and V_{max} values for this transporter were calculated as 134 ± 36 μM and 0.14 ± 0.03 pmol/10^7 parasites/s (n = 4).

To find out whether separate transporters are involved in the uptake of inosine and adenosine, [³H]adenosine transport was measured in the presence of increasing concentrations of unlabelled inosine and [³H]inosine transport was measured in the presence of unlabelled adenosine.

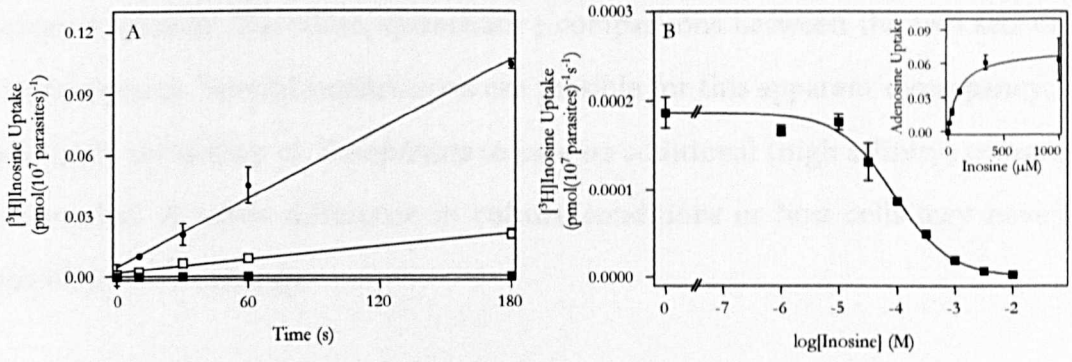


FIGURE 7.5 Transport of [^{3}H]inosine in extracellular tachyzoites. **A.** Uptake of 0.1 μM [^{3}H]inosine (■; 1.6 ± 0.2 x 10^{-6} pmol(10^7 parasites)$^{-1}$s^{-1}), 1 μM [^{3}H]inosine (□; 1.1 ± 0.1 x 10^{-4} pmol(10^7 parasites)$^{-1}$s^{-1}) and 10 μM [^{3}H]inosine (●; 5.7 ± 0.3 x 10^{-4} pmol(10^7 parasites)$^{-1}$s^{-1}) measured over various intervals between 0 and 180 s. **B.** [^{3}H]Inosine uptake (1 μM) was measured over 120 s in the presence of 0 – 10 mM unlabeled inosine (IC_{50} = 91.7 μM). Inset shows conversion to Michaelis-Menten plot (K_m = 100 μM; V_{max} = 0.072 pmol(10^7 parasites)$^{-1}$s^{-1}).

The results, depicted in Figure 7.6, show that inosine does inhibit adenosine transport, and *vice versa*, but in a biphasic manner, revealing a high affinity transport capacity for the two purine nucleosides. The presence of high affinity [^{3}H]adenosine and [^{3}H]inosine transport was confirmed at label concentrations of 25 and 100 nM, respectively (Figure 7.7*A,B*). K_m and K_i values for the high and low affinity transporters of [^{3}H]adenosine and [^{3}H]inosine are given in Table 7.1. The data are entirely consistent with there being two transporters, both the previously described low affinity nucleoside transporter TgAT1 (Schwab, *et al.*, 1995) and an additional high affinity transporter, designated TgAT2. Both transport adenosine as well as inosine: the K_m values for [^{3}H]adenosine transport are very similar ($P > 0.05$; unpaired *T*-test) to the K_i values for adenosine using [^{3}H]inosine as permeant, and this is also true for the reverse experiment. It should be noted that the data in Figure 7.6 were obtained much later than those in Figures 7.4 and 7.5. In the first set of experiments, we observed a rate consistent with Figure 7.4, very consistently, leading to a V_{max} estimate for adenosine of 0.066 ± 0.002 pmol(10^7 cells)$^{-1}$s^{-1} – note the small error over three experiments. In a later series, using stabilates from the same strain, we consistently observed a much higher rate of transport at low permeant concentrations and clear evidence for two

transporter systems. Therefore, quantitative comparisons between the two sets of data are inappropriate. Several explanations are possible for this apparent discrepancy, most prominently the ability of *Toxoplasma* to express additional (high affinity) transporters when needed. A subtle difference in culture conditions or host cells may have been responsible for the change.

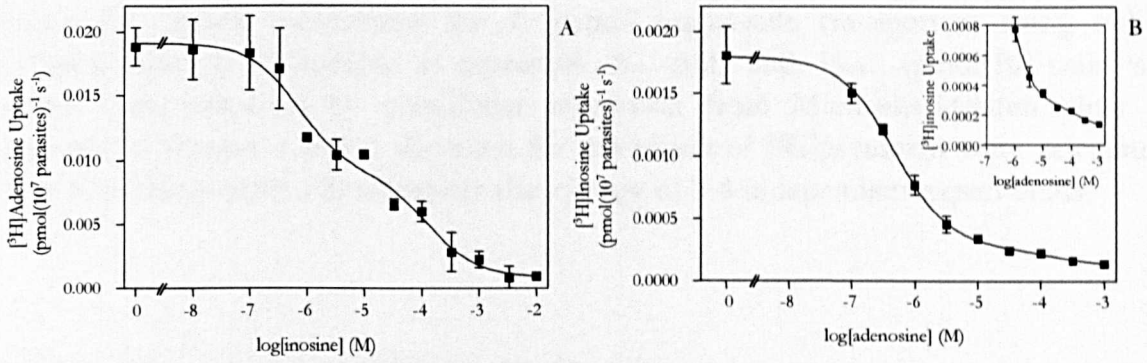


FIGURE 7.6 Adenosine and inosine compete for the same transporters in *T. gondii* tachyzoites. **A.** Inhibition of 1.25 μM [³H]adenosine by up to 10 mM unlabelled inosine was shown to be biphasic by non-linear regression (P=0.0011), with IC_{50} values of 0.70 and 127 μM. **B.** Transport of 1 μM [³H]inosine was inhibited by adenosine in a biphasic way (P=0.018), with IC_{50} values of 0.49 and 174 μM, respectively. Inset shows the same data on a different scale.

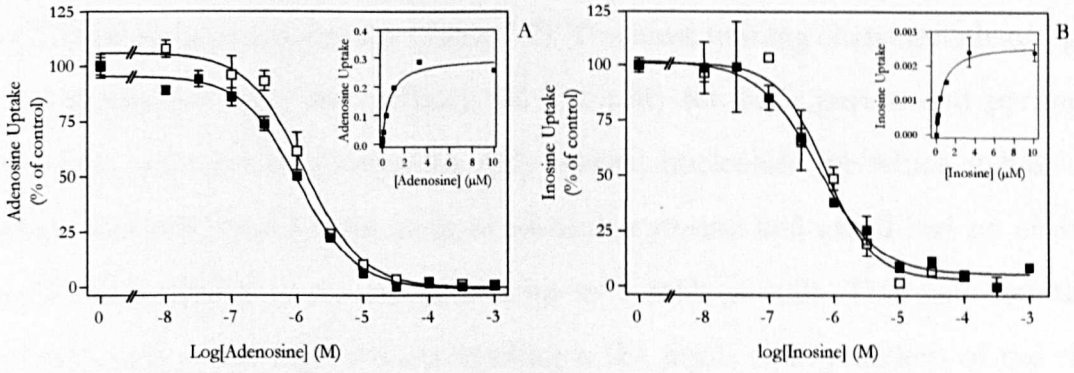


FIGURE 7.7 High affinity transporters for adenosine and inosine. **A.** Uptake of 25 nM [³H]adenosine (expressed as percentage of control - no inhibitor) was inhibited by up to 1 mM unlabeled adenosine (■, IC_{50} = 1.1 μM) or inosine (□, IC_{50} = 1.3 μM). Inset shows the conversion to Michaelis-Menten plot with K_m = 0.61 μM and V_{max} = 0.30 pmol(10⁷ parasites)⁻¹s⁻¹. **B.** Uptake of 100 nM [³H]inosine in the presence or absence of various concentrations of unlabelled inosine (■, IC_{50} = 0.56 μM) or adenosine (□, IC_{50} = 0.76 μM). Michaelis-Menten plot (*inset*) yielded K_m (0.68 μM) and V_{max} (0.0026 pmol(10⁷ parasites)⁻¹s⁻¹) values.

	TgAT1		*TgAT2*	
	[^{3}H]adenosine	[^{3}H]inosine	[^{3}H]adenosine	[^{3}H]inosine
K_m	105 ± 22	134 ± 36	0.49 ± 0.12	0.77 ± 0.20
V_{max}	0.066 ± 0.002	0.14 ± 0.03	0.17 ± 0.04	0.0048 ± 0.0026
K_i (adenosine)		88 ± 35		0.40 ± 0.12
K_i (inosine)	105 ± 19		0.28 ± 0.11	

TABLE 7.1 Kinetic parameters for *T. gondii* nucleoside transporters using either [^{3}H]adenosine or [^{3}H]inosine as permeant. K_m (μM) and V_{max} (pmol(10^7 cells)$^{-1}$s^{-1}) values were obtained by non-linear regression from Michaelis-Menten plots as depicted in Figures 1 and 3. K_i values for inhibition of [^{3}H]permeant were calculated from IC_{50} values (μM). All values are the average of 3-4 independent experiments.

7.2.3 TgAT2 is a broad specificity nucleoside transporter

Whereas the low affinity transporter is most likely the same adenosine transporter described earlier by Schwab *et al.* (1995) and cloned by Chiang *et al.* (1999), a high affinity nucleoside transport has not previously been described for *T. gondii*. We therefore undertook a thorough analysis of the substrate selectivity of TgAT2, using 50 nM [^{3}H]adenosine as a permeant (Table 7.2). The most striking characteristic of TgAT2 is that it displays very high affinity (K_i < 2 μM) for both purine and pyrimidine nucleosides, with cytidine being the only natural nucleoside for which it has lower affinity. However, nucleobases such as adenine, cytosine and uracil had no effect on [^{3}H]adenosine uptake at concentrations up to 1 mM (n = 3). This indicates that a significant part of the high affinity binding is the result of interactions of the ribose moiety with amino acid residues in the TgAT2 binding pocket. These interactions appear to consist of hydrogen bonds to the 3' and 5' hydroxyl groups of the nucleosides, as the K_i values for 3'- and 5'-deoxythymidine were >200-fold higher than those for thymidine. This translates into an apparent loss of Gibbs free energy for the interaction of the substrate with the transporter of 12.9 and 16.8 kJ/mol, respectively (Table 7.2). It is highly unlikely that the 2' hydroxyl group contributes to substrate binding, as

thymidine, which lacks a 2' hydroxyl group, displays the same affinity for TgAT2 as does uridine, which does have a hydroxyl at this position. This observation also suggests that the only other difference between uridine and thymidine, the methyl group at position 5 of the pyrimidine ring, does not make an important contribution to the binding energy of the nucleoside.

Compound	*Ki* (μM)	ΔG^0 (kJ/mol)
Purine nucleosides		
Adenosine	0.49 ± 0.12	-36.0
Inosine	0.28 ± 0.11	-37.4
Guanosine	1.5 ± 0.3	-33.2
Xanthosine [a]	556 ± 53	-18.8
3-Deazaadenosine [a]	400 ± 45	-19.4
7-Deazaadenosine	0.24 ± 0.04	-37.8
AraA	2.9 ± 0.8	-31.6
Pyrimidine nucleosides		
Uridine	1.5 ± 0.4	-33.2
Thymidine	1.3 ± 0.5	-33.6
Cytidine	32 ± 10	-25.7
2-Thiouridine	0.68 ± 0.06	-35.2
4-Thiouridine	0.40 ± 0.03	-36.5
3-deazauridine [a]	360 ± 78	-19.7
3'-deoxythymidine	280 ± 82	-20.3
5'-deoxythymidine	1040 ± 210	-17.0
Ribose [a]	22500 ± 7800	-9.4

TABLE 7.2 TgAT2 inhibition constants (*Ki*) and Gibbs Free Energy (ΔG^0) for various nucleosides. *Ki* or *Km* values were calculated form IC_{50} values obtained from inhibition curves featuring a minimum of 6 points over the relevant range, using equation 1. [^{3}H]Adenosine concentration was 50 nM throughout. Gibbs free energy was then obtained using equation 2, as described in Chapter two. [a] Estimated from partial curve, giving >50% inhibition. Hill slope was assumed to be –1, and eventual inhibition was taken to be equal to that of 1 mM adenosine.

Even though the ribose moiety contributes very strongly to nucleoside binding by TgAT2, ribose itself only weakly inhibited [^{3}H]adenosine uptake. The estimated K_i value was around 22 mM. This is some 25 kJ/mol less binding energy than with adenosine or uridine and the difference must be attributed to binding of the purine or pyrimidine base group. The keto groups at positions 2 and 4 of uridine were not involved in interactions with the transporter, as K_i values for 2-thiouridine and 4-thiouridine were slightly lower than for uridine (Figure 7.8*A*). The slightly higher affinity may be related to the protonation state of N3, as the thione group is less basic than the keto group. As a result, protonated N3 is a slightly better H-bond donor in either thiouridine. The importance of this is readily confirmed by the very low affinity of TgAT2 for 3-deazauridine (Figure 7.8*A*). The $\delta(\Delta G^0)$ of 13.5 kJ/mol accounts for most of the apparent bond energy of the pyrimidine ring. The remainder must, by default, be the result of π–π stacking with an aromatic amino acid residue, as the uracil moiety contains no other functional groups capable of forming interactions of sufficient energy with the TgAT2 binding pocket. The significantly lower affinity for cytidine than for uridine or thymidine is explained by the greatly reduced level of protonation at N3, which changes this residue from a hydrogen bond donor to an H-bond acceptor.

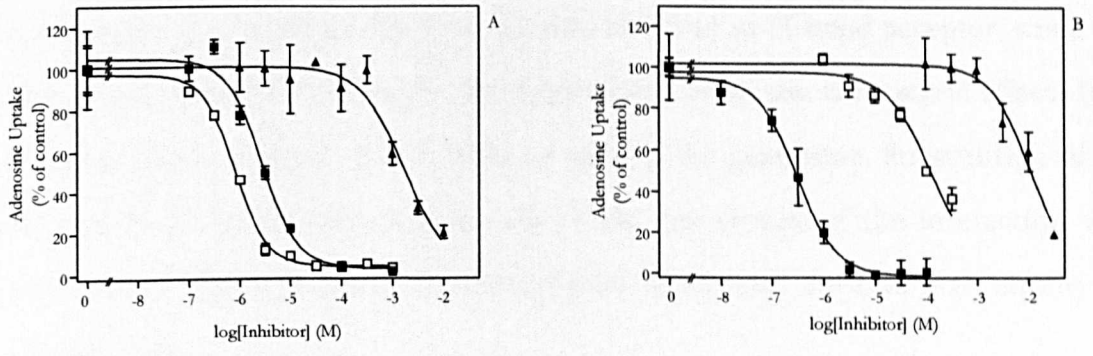


FIGURE 7.8 Substrate selectivity of TgAT2. Uptake of 50 nM [^{3}H]adenosine by extracellular tachyzoites was measured in the presences of various concentrations of potential inhibitors and expressed as percentage of control, defined as [^{3}H] adenosine uptake in the absence of inhibitor. A. Pyrimidines: uridine (■), 2-thiouridine (□) and 3-deazauridine (▲). B. Purines and ribose: 7-deazaadenosine (■), 3-deazaadenosine (□) and ribose (▲). In the case of incomplete inhibition plots, the Hill slope was assumed to be –1 and eventual inhibition 100%, being equal to inhibition by 1 mM adenosine.

Purines appear to be similarly bound by H-bonds to the 3' and 5' hydroxyl groups, as well as a single H-bond to a ring nitrogen residue and π–π stacking. The unprotonated N3 residue forms the main H-bond with the TgAT2 binding pocket, as evident from the almost 1000-fold higher K_i value for 3-deazaadenosine than for adenosine (Figure 7.8*B*). This constitutes an apparent loss of 16.6 kJ/mol in Gibbs free energy. Assuming a similar positioning of the purine and pyrimidine nucleosides in the transporter, stabilised by the strong binding of the ribose moiety present in both, it is unsurprising that both should be capable of interaction with the same aromatic residue for the energetically favourable π–π stacking (Figure 7.9). There was no evidence for further interactions of TgAT2 with other parts of the purine ring. 7-Deazaadenosine (tubercidin) displayed virtually the same binding energy as adenosine (Figure 7.8*B*), ruling out any contribution from N7. The lack of discrimination between aminopurines and oxopurines (*i.e.* adenosine and inosine) appears to rule out significant interactions with either the amino or keto groups at position 6, or with N1 (whether protonated as in oxopurines or unprotonated as in aminopurines). However, the amino group at position 2 appears energetically unfavourable, given the slightly lower affinity for guanosine (Table 7.2). Neither is an H-bond acceptor more favourable at this position given the $\delta(\Delta G^0)$ of 18.8 kJ/mol of xanthosine, compared with inosine. This large loss of interaction energy results mostly from the loss of N3 as an H-bond acceptor, since this residue is protonated in xanthosine, but additionally from the same steric effects from the substitution at position 2 that reduced affinity for guanosine. Substitution of the ribose moiety for arabinoside reduces the Gibbs free energy of the interaction with TgAT2, by 4.4 kJ/mol, but adenine arabinoside (AraA) still displays high affinity for this transporter.

The model for the interactions of TgAT2 with uridine and adenosine is shown in Figure 7.9*A*. It seems reasonable to predict that the π–π stacking occurs mostly with the imidazole part of the purine ring, since the aromatic amino acid must be equally well positioned for optimal energy gain with the pyrimidine ring.

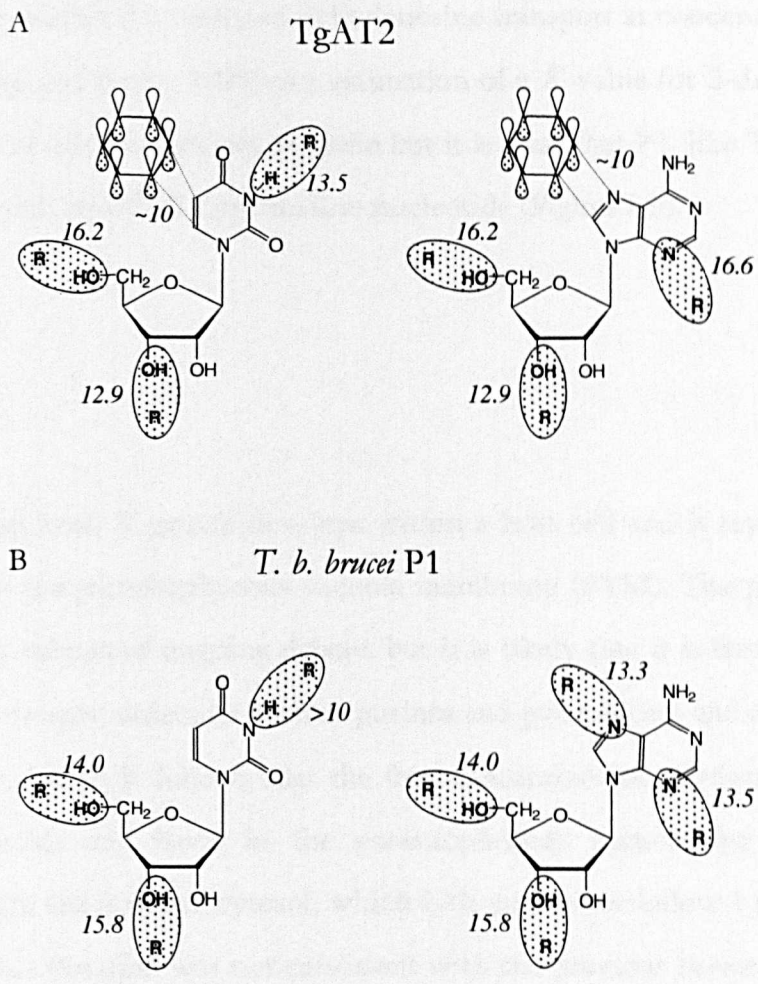


FIGURE 7.9 Model for interactions between (**A**) the TgAT2 or (**B**) TbbP1 binding pocket and adenosine or uridine. Shaded areas depict areas of interaction with amino acid residues (R) in the transporter binding pocket, most probably H-bonds. An aromatic ring with π–orbitals is schematically included to depict π–π stacking with the purine or pyrimidine ring. Apparent ΔG^0 for the interactions is given in kJ/mol. The model for TbbP1 was adapted from (De Koning and Jarvis, 1999).

The TgAT2 mechanism for binding of adenosine is very close to that used by the *T. b. brucei* P1 transporter (De Koning and Jarvis, 1999), depicted in Figure 7.9*B* for comparison. The sole difference between the two binding pockets appears to be the change of the aromatic residue of TgAT2 to a hydrogen bond donor in P1. Indeed, this change, and the resultant absence of π-stacking to bind the pyrimidine ring, explains the low affinity of P1 for pyrimidine nucleosides (De Koning and Jarvis, 1999). However, P1 does bind uridine, with a K_i value of 830 ± 86 μM, but not 3-deazauridine,

which does not inhibit P1-mediated [^{3}H]adenosine transport at concentrations up to 10 mM (De Koning and Jarvis, 1999). An estimation of a K_i value for 3-deazauridine, and $\delta(\Delta G^0)$ relative to uridine, can not be given but it is clear that P1, like TgAT2, can form a strong bond with N(3)H of a pyrimidine nucleoside (Figure 7.9).

7.3 Discussion

In a mammalian host, *T. gondii* develops within a host cell and is separated from the host cytosol by the parasitophorous vacuole membrane (PVM). The precise nature of the PVM is the subject of ongoing debate, but it is likely that it is freely permeable to low-molecular weight molecules such as purines and pyrimidines and their nucleosides (Schwab, *et al.*, 1994). It follows that the free concentrations of adenosine and other purine nucleosides and bases in the parasitophorous vacuole are similar to the concentrations in the host cell cytosol, which is thought to be below 1 μM (Plagemann, *et al.*, 1988). This situation was not consistent with the previous literature which states that adenosine is the main purine source for *T. gondii* (Krug, *et al.*, 1989) and that tachyzoites express just a single, low affinity adenosine transporter, with a K_m > 100 μM (Schwab, *et al.*, 1995; Chiang, *et al.*, 1999). This paradox seemed to be resolved with the discovery of a NTPase that is secreted into the parasitophorous vacuole (PV) in large amounts by the tachyzoites (Asai, *et al.*, 1983; Sibley, *et al.*, 1994; Asai, *et al.*, 1995) and which was believed to produce large quantities of free adenosine within the PV by degrading the ATP entering the PV from the host cell's cytosol (Schwab, *et al.*, 1995). However, it is now known that only a small proportion of the NTPase is in an active form (Silverman, *et al.*, 1998) and it yields mono-phosphorylated nucleotides rather than nucleosides (Asai, *et al.*, 1995). AMP is a very poor purine source for tachyzoites as it cannot be taken up without prior dephosphorylation (Schwartzman and Pfefferkorn, 1982; Ngo, *et al.*, 2000). Ngo, *et al.* (2000) were not able to detect, either in the PV itself or on the *T. gondii* plasma membrane, the ecto-5'-nucleotidase activity

required to mediate this dephosphorylation. Moreover, activation of the NTPase leads to rapid lysis of the host cell (Silverman, *et al.*, 1998). Thus the overall evidence suggests that the enzyme does not play any significant role in purine salvage by the intracellular tachyzoite.

Toxoplasma, like all protozoan parasites studied to date except for the opportunistic *Acanthamoeba* (Hassan and Coombs, 1986), is a purine auxotroph (Perrotto, *et al.*, 1971; Schwartzman and Pfefferkorn, 1982) and the absence of a credible source of purines that could be salvaged by the low affinity transporter TgAT1 prompted us to re-investigate purine transport in tachyzoites. Our finding that *T. gondii* expresses high affinity transporters for purine nucleosides and for hypoxanthine/guanine explains how the parasite is able to salvage efficiently the low levels of free purines that are thought to be present in the PV.

The low affinity transport activity also described here is almost certainly the TgAT transporter recently cloned by Chiang, *et al.* (1999). This transporter displayed a K_m of 114 μM and was sensitive to inosine. In 1995, Schwab and colleagues described a similar inosine-sensitive adenosine transporter in *T. gondii* tachyzoites, with a K_m of 120 μM, although it was unclear whether a second inosine transporter was also expressed. These authors also reported that adenine and hypoxanthine inhibited adenosine transport and suggested that this transporter mediated both nucleoside and nucleobase uptake. However, inhibition by these bases did not exceed 50% at 1.6 mM (Schwab, *et al.*, 1995) and the identification of a high affinity hypoxanthine transporter, which is reported here, suggests that the low affinity adenosine transporter is unlikely to make any major contribution to nucleobase uptake in tachyzoites.

TgAT1 was cloned by insertional mutagenesis that resulted in resistance to AraA (Chiang, *et al.*, 1999). Chiang and colleagues reported that a single insertion event, which resulted in a loss of TgAT1 activity, conferred AraA resistance. These findings appear to differ from those of the current study, which demonstrate that tachyzoites

are capable of expressing a second transporter that should be readily able to mediate AraA uptake. There are several possible explanations for these apparently contradictory findings. It is possible that TgAT2 does not in fact transport AraA, though it is inhibited by it. However, this would not explain the apparent lack of [^{3}H]adenosine transport by the mutant line (Chiang, *et al.*, 1999; Ngo, *et al.*, 2000). It is conceivable that TgAT1 and TgAT2 are products of the same gene, produced by alternative splicing or post-translational modifications. If this is the case, expression of active TgAT2 may be controlled by environmental factors and may not have occurred under the culture conditions and the type of host cells used in Chiang *et al.*'s study. A single amino acid substitution between allelic isoforms of the *candida albicans* concentrative nucleoside transporter CaCNT produced distinct high- and low-affinity nucleoside transporter activities (Slugoski, *et al.*, 2004). Moreover, it may be possible that a single insertion event is capable of disrupting two adjacent genes and that such happened with the two nucleoside transporters. Further studies on the regulation of the expression of these transporters, and/or the cloning of TgAT2, will be required to resolve this issue.

The results of Schwab *et al.* (1995) and Chiang *et al.* (1999) showed that TgAT1 transports only purine nucleosides, as the transporter was insensitive to inhibition by sub-mM concentrations of nucleobases or pyrimidine nucleosides. In contrast, TgAT2 displays high affinity for all natural pyrimidine and purine nucleosides. The combination of very high affinity and broad specificity has not been described before for a protozoan nucleoside transporter. On the basis of sequence homology, protozoan nucleoside transporters characterised to date have been classified as belonging to the ENT family, which includes the human equilibrative nucleoside transporters (Hyde, *et al.*, 2001). However, like the *Leishmania donovani* (Vasudevan, *et al.*, 1998; Carter, *et al.*, 2000b) and *Trypanosoma brucei* nucleoside transporters (Carter and Fairlamb, 1993; De Koning, *et al.*, 1998; De Koning, and Jarvis, 1999), TgAT2 displays 2-3 orders of magnitude higher affinity for its substrates than do any of the mammalian family members. Functionally, TgAT2 most closely resembles the human sodium-dependent nucleoside transporter hCNT3 in that this transporter also recognises all physiological

nucleosides with similar affinity (apparent K_m values 15-52 μM) (Ritzel, *et al.*, 2001b; Toan, *et al.*, 2003).

The very broad substrate specificity, the high affinity and the high capacity of TgAT2 means that, like hCNT3 (Ritzel, *et al.*, 2001b; Smith, *et al.*, 2005), it could be an efficient conduit for the uptake of various therapeutic nucleosides. We have therefore further investigated the mechanisms that allow TgAT2 to bind oxopurines, aminopurines and pyrimidines with sub-μM affinity, using the same techniques that enabled the modelling of the transporter-substrate interactions of the *T. brucei* nucleoside transporters (De Koning and Jarvis, 1999) and the human, trypanosome and *Leishmania major* nucleobase transporters (Wallace, *et al.*, 2002; Papageorgiou, *et al.*, 2005; Chapter three). The low affinities for 3'- and 5'-deoxythymidine, relative to thymidine, clearly demonstrate the importance of these hydroxyl groups for optimal interactions with the transporter, even though it cannot be affirmed whether they function as a hydrogen bond acceptor, donor, or both. In contrast, the 2' hydroxyl does not contribute to transporter recognition, as thymidine has an equal affinity for TgAT2 as uridine. The high energy of the two ribose hydrogen bonds ensures that the ribose moiety as a whole has a major contribution to the very high affinity of TgAT2 for nucleoside substrates and is sufficient to explain the lack of interaction with any nucleobases at concentrations below 1 mM. It also helps to explain the broad specificity for physiological nucleosides including 2'-deoxynucleotides. It shares this feature, as well as a strong H-bond with N3 of the purine ring, with the *T. b. brucei* P1 transporter (De Koning and Jarvis, 1999). However, whereas the latter transporter is purine-specific, TgAT2 also efficiently transports pyrimidines. This major difference in substrate specificity could be due to a single substitution of a H-bond donor in the permeant binding pocket to an aromatic amino acid residue. Whereas P1 forms a H-bond with an apparent energy of –13 kJ/mol with N7 of adenosine, TgAT2 displayed equal affinity for adenosine and 7-deazaadenosine (tubercidin). Neither is it likely that TgAT2 forms H-bonds with N1 or with substitutions at position 6, as it does not

discriminate between oxopurines (N1 in lactam form) and aminopurines (pyrimidine-like N1). The postulation of π–π stacking is therefore the result of exclusion of all other possibilities and the necessity of accounting for ~10 kJ/mol in the Gibbs free energy between 3-deazaadenosine and ribose. The situation is even clearer for the binding of the pyrimidine nucleosides, as the pyrimidine ring of uridine is bound only through a hydrogen bond at N(3)H: substitution of either keto group by thione failed to reduce affinity for TgAT2. The difference in Gibbs free energy between binding uridine or ribose was 23.8 kJ/mol, and only 13.5 kJ/mol could be attributed to the H-bond with N(3)H. This leaves again ~10 kJ/mol for π-stacking. The importance of a H-bond donor at position 3 is also illustrated by the relatively low affinity for cytidine, the predominant tautomeric form of which has an unprotonated N3. Similar π-stacking energies have been reported for the *T. b. brucei* P2 aminopurine transporter (De Koning and Jarvis, 1999) and the human FNT1 nucleobase transporter (Wallace, *et al.*, 2002).

T. gondii tachyzoites also express a high affinity transporter for hypoxanthine, which may also transport guanine and xanthine, but not adenine, uracil or nucleosides. This transporter, designated TgNBT1, is the first high affinity nucleobase transporter described in an apicomplexan parasite. The only previous report on nucleobase transport by an apicomplexan parasite was for the *Plasmodium falciparum* PfENT1 transporter, with K_i values >300 μM (Parker, *et al.*, 2000). Carter *et al.* (2000a) described an almost identical nucleoside transporter (PfNT1), but reported that it was not significantly inhibited by a 100-fold excess of purine and pyrimidine bases. However, high affinity hypoxanthine transporters have been detected in trypanosomatid parasites, including *T. brucei* (De Koning and Jarvis, 1997a; De Koning and Jarvis, 1997b; Burchmore, *et al.*, 2003), *Crithidia luciliae* (Day and Gero, 1997), *L. major* (Al-Salabi, *et al.*, 2003), *L. braziliensis panamensis* (Hansen, *et al.*, 1982), and *L. mexicana* (Al-Salabi and De Koning, 2005) as well as in many other micro-organisms and metazoans (De Koning and Diallinas, 2000). The narrow specificity of TgNBT1 for

just hypoxanthine and guanine is, however, unusual. Other eukaryotic nucleobase transporters have predominantly been reported as broad-specificity purine transporters or oxopurine/oxopyrimidine transporters (De Koning and Diallinas, 2000). The uptake of hypoxanthine indeed appears to show that uptake rates are not saturating at the expected concentration of ~1 μM. In addition, there appears to be a second inhibitory phase in the inhibition experiment, also indicative of a low affinity hypoxanthine uptake system. The nature of this secondary, low affinity system was not investigated in this study, but it is likely that it is mediated by one of the two broad specificity nucleoside transporters, displaying low affinity for nucleobases and capable of transporting them.

The absence of detectable adenine transport activity was surprising given the ability of tachyzoites to incorporate [^{3}H]adenine into nucleic acids (Krug, *et al.*, 1989). However, Krug *et al.* (1989) postulated that adenine is first deaminated. Thus the use of adenine may well also involve TgNBT1, although the required deaminase would need to be located in the PV or at least on the *T. gondii* plasma membrane. Schwab *et al.* (1995) did report that [^{3}H]adenine is taken up by tachyzoites, but did not investigate whether this was saturable and so involved a transporter.

This study has demonstrated that TgAT2 binds nucleoside analogues, such as tubercidin (7-deazaadenosine) and AraA, with high affinity, though formal proof that these potential antimetabolites are indeed transported by TgAT2 will require studies with radiolabelled compounds. The model presented here predicts that the same therapeutic nucleosides efficiently transported by hCNT3 (Ritzel, *et al.*, 2001a; Ritzel, *et al.*, 2001b), including gemcitabine, 5-fluoro-2'-deoxyuridine, fludarabine, cladrabine and, to a slightly lesser extend, zebularine (Figure 7.10), will be excellent permeants for TgAT2. While this makes TgAT2 a very promising conduit for therapeutic agents, it must be remembered that in order to be effective against the growing parasites these agents would need to be able cross the host cell membrane and so enter the PV. It was

demonstrated that nitrobenzylthioinosine (NBMPR), an inhibitor of nucleoside transport in mammalian cells, was transported by intracellular *Toxoplasma gondii* grown in human foreskin fibroblast cells. However, under the same conditions, uninfected fibroblast cells did not transport NBMPR (Al Safarjalani, *et al.*, 2003). These findings of purine nucleoside transport in *T. gondii* may aid in the identification of new promising antitoxoplasmic drugs. Further studies on transport of potentially therapeutic nucleosides by the various human nucleoside transporters should provide additional insights as to whether such nucleoside antimetabolites could be effective agents against toxoplasmosis and similar infections, although the possibility that infection by *Toxoplasma* alters the permeability of the host cell, as is the case with infections with *Plasmodium* (Kirk, 2001), should not be overlooked.

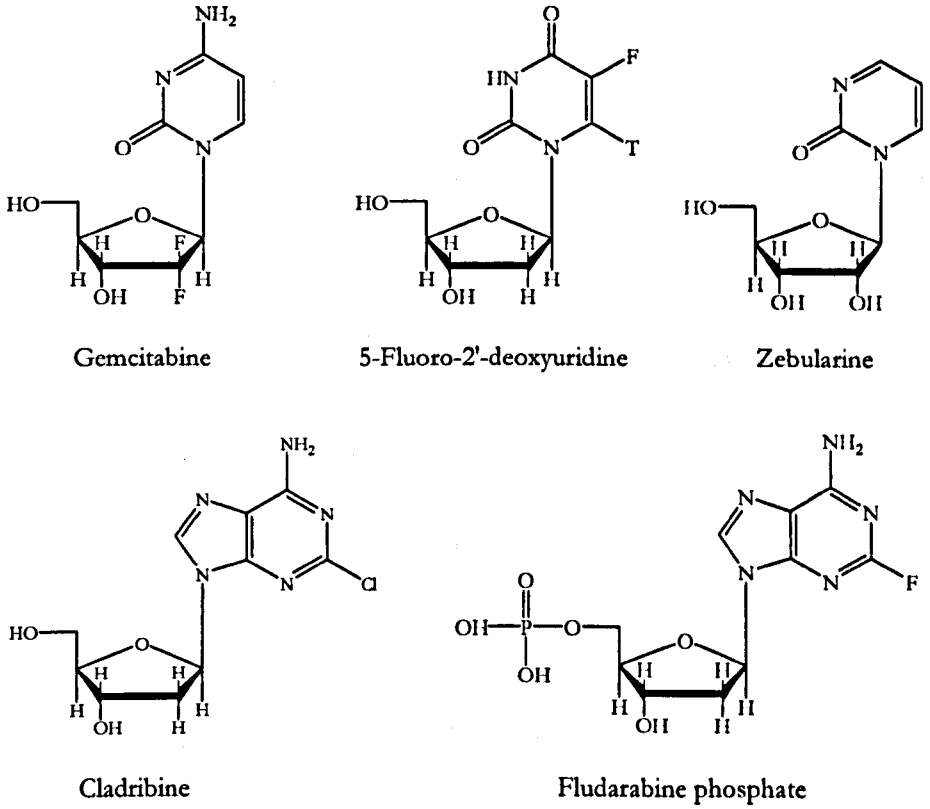


FIGURE 7.10 Structure of some therapeutic nucleosides, which can be excellent permeants for TgAT2.

Chapter Eight

GENERAL DISCUSSION

The rationale behind this study of purine salvage in protozoa is that parasitic diseases have been the cause of great suffering and death throughout recorded history. These diseases, which include leishmaniasis and trypanosomiasis, are among the most neglected in the world, affecting millions of the world's poorest people and attracting little or no interest from pharmaceutical companies. Furthermore, other parasitic diseases such as toxoplasmosis are considered as a major health problem for immunocompromised individuals such as AIDS patients, and unborn children of infected women.

Chemotherapy is currently the only option for the control and treatment of parasitic infections as no vaccines are currently available to prevent or control the spread of these diseases. Controlling the invertebrate vector of these parasites, such as the mosquito, sandfly and tsetse fly, is very difficult due to insecticide resistance, concerns regarding environmental damage and lack of adequate infrastructure and funds to apply existing vector control methods on the required scale.

Treatment and control of parasitic diseases of both the human and the veterinary condition is adversely affected by many different issues. Toxicity is one of the problems associated with the current drugs. For example, Melarsoprol, an arsenical compound, is the drug of choice for late-stage African trypanosomiasis, but often induces serious, sometimes fatal side effects. Pentavalent antimonial compounds, either sodium stibogluconate (Pentostam) or meglumine antimoniate (Glucantime) which are still the first choice drugs in the treatment of all forms of leishmaniasis after many decades, have many severe side effects (Wyler and Marsden, 1984; Navin, *et al.*, 1992; Olliaro and Bryceson, 1993; Hepburn, *et al.*, 1993; Gasser, *et al.*, 1994; McBride, *et al.*, 1995). Pyrimethamine (Daraprim), an antimalarial drug, is used to treat toxoplasmosis, but also has potential side effects, including bone marrow suppression, leukopenia, anemia, thrombocytopenia and liver toxicity (Schwartzman, 2001).

Resistance to common drugs is a severe and increasing problem in the treatment of almost all parasitic diseases. The lack of response to pentavalent antimonials in visceral leishmaniasis has been a problem for many years and is increasing worldwide (Ouellette and Papadopoulou, 1993; Guerin, *et al.,* 2002). The increasing incidence of resistance to drugs used to treat African trypanosomiasis has become a real problem, especially in the countries with highest transmission rates (Peregrine, 1994). For example, treatment failure in melarsoprol-treated patients is on the increase, particularly in Sudan, Angola, Democratic Republic of the Congo and Uganda (Legros, *et al.,* 1999). The development of resistance to isometamidium chloride has been reported in both East and West Africa (Peregrine, 1994). Treatment failures have been reported for toxoplasmosis when combinations of pyrimethamine-sulfadiazine and pyrimethamine-clindamycin were used (Katlama, *et al.*, 1996).

The high cost of some of the current drugs for parasitic diseases is also a major problem because most of the people suffering from these infections are in poor counties. Melarsoprol is very expensive as a complete course of treatment costs US$ 50. However, at US$ 250 a patient, DFMO is just too expensive to use as treatment of late stage sleeping sickness victims in Africa (Aksoy, 2003), and is used strictly as a last resort for melarsoprol-refractory patients. In addition, the I.V. administration of DFMO is extremely difficult under field conditions.

More effective and less costly therapeutic agents are therefore urgently needed as the situation of parasitic diseases deteriorates and millions of people are at imminent risk. The researchers' goal is to translate basic research discoveries into candidate drugs ready for clinical trials. In theory, it should be possible after understanding the physiology and biochemistry of these pathogens, to look for weak points that can be exploited for chemotherapeutic intervention. The science of rational chemotherapy began in earnest with the work of Paul Ehrlich who invented the term "chemotherapy" and advanced the concept of the "magic bullet", an agent of low molecular weight that

selectively attacks the foreign invading organisms curing the disease without causing damage to the host (Bryant and Behm, 1989). One general approach to the development and design of rational drugs is to identify fundamental biochemical differences between parasite and host, such as the inability of protozoan parasites, including *Leishmania*, *Trypanosoma*, and *Toxoplasma*, to synthesize purines *de novo* (Berens, *et al.*, 1995).

The aim of this project, therefore, is to identify and characterize nucleobase and nucleoside transporters involved in purine salvage. These carriers are of considerable pharmacological interest since they are considered as promising targets for the development of antiparasitic chemotherapy. Studying purine transporters in these parasites is very useful to understand the biochemistry of these parasites as these transporters play essential roles in their nutrition at the different stages of their life-cycle. The identification and functional characterisation of these transporters can also provide a target for chemotherapy by two approaches. First, by blocking the uptake of essential nutrients like purines and developing inhibitors of these transporters. However, using purine transporters as chemotherapeutic target in this way will be almost impossible. Second, by the selective delivery of cytotoxic molecules (purines or non-purines) to parasites using these transporters. Almost all current trypanocides act selectively on the parasitic rather than the host cells because they are selectively accumulated by the parasite as a result of differences in plasma membrane transporters (De Koning, 2001a; El Kouni, 2003). This approach, to rationally design new trypanocides that are selectively accumulated through purine transporters, has already been applied with considerable success in recent years (Barrett, *et al.*, 2000; Rodenko and De Koning, unpublished).

Analogues of purines, when used alone or in combination with other drugs, are being evaluated as chemotherapeutic agents and are effective against almost any fast growing cells (El Farrash, *et al.*, 2003; Namazi, 2004). For example, allopurinol, a hypoxanthine

analogue, has been evaluated in the treatment of *Leishmania braziliensis* infection and Chagas disease (Cohn and Gottlieb, 1997). In addition, allopurinol has been used to treat leishmaniasis alone (Pfaller and Marr, 1974; Marsden, *et al.*, 1984) or combined with other drugs (Martinez and Marr, 1992; Martinez, *et al.*, 1997; Denerolle and Bourdoiseau, 1999; Momeni, *et al.*, 2002; Pasa, *et al.*, 2005). Among many other examples of apparently efficacious purine analogues, 9-deazainosine displayed promising therapeutic indexes in *L. donovani*-infected hamsters (Berman, *et al.*, 1987).

We have extensively investigated purine nucleobase and nucleoside transporters in *Leishmania* spp., *Trypanosoma* spp., and *Toxoplasma gondii*. These organisms are important as chemotherapeutic targets as they are responsible for the tragic consequences of the diseases mentioned above and are also a good model for the study of transporter function. These parasites are easily grown in suspension and to high densities, their genomes have been sequenced and published, their genes do not have introns and their biology and life cycle have been very well studied.

At the start of this project, there was nothing known about nucleobase transporters in *Leishmania* species. We first identified a single transporter, designated LmajNBT1 in the promastigote forms of *L. major* (Chapter three). This transporter was the first nucleobase transporter to be functionally characterised and was shown to possess a high affinity for all physiological purine bases and moderately high affinity for allopurinol. Studies with [^{3}H]allopurinol confirmed that this transporter is its sole route of entry into promastigotes. Despite numerous studies on allopurinol metabolism in kinetoplastids (Marr, *et al.*, 1978; Nelson, *et al.*, 1979a; Nelson, *et al.*, 1979b; Marr, 1983; Marr and Berens, 1983), this study was the first to address the mechanism of allopurinol uptake by the parasite, which is important in understanding the selectivity of the drug as well as the potential for the development of resistance for allopurinol. Indeed, there are indications that resistance to this analogue could be induced quite easily (Cavaliero, *et al.*, 1999; Kamau, *et al.*, 2000).

Earlier studies reported that *L. donovani* promastigotes also possess two purine nucleoside transporters, designated LdNT1 and LdNT2. The first transported adenosine and its analogues, formycin A and tubercidin, as well as the pyrimidine nucleosides, while the second carried inosine, guanosine, and their analogues including formycin B (Iovannisci, *et al.,* 1984; Aronow, *et al.,* 1987). These findings were confirmed when two closely linked genes, *LdNT1.1* and *LdNT1.2*, were identified. The LdNT1.1 and LdNT1.2 transporters mediated the uptake of adenosine and the pyrimidine nucleosides with high affinity (Vasudevan, *et al.,* 1998). In addition, a single gene, *LdNT2*, encoding the inosine-guanosine transporter function LdNT2, has been cloned (Carter, *et al.,* 2000b). It has also been suggested that the transport of adenosine, but not inosine, seemed to have different activities in various strains of *L. donovani* (Ogbunude, *et al.,* 1991) and *L. major* (Baer, *et al.,* 1992) promastigotes, which may be related to the existence of the two gene copies for NT1. Two adenosine transporters, T1 and T2, have also been reported in *Leishmania donovani* amastigotes (Ghosh and Mukherjee, 2000). T1 is an adenosine/pyrimidine transporter and was very similar to the LdNT1 activity which was originally detected in promastigotes. T2, the second adenosine transporter, is inhibitable by inosine and exclusively present in amastigotes. It is thus evident that multiple purine transporters (isoforms) exist in all stages of *Leishmania*, and that developing a single inhibitor to block them all will be exceedingly difficult.

The next step in this study was to assess whether nucleobase transport activity is different in various life cycle stages of *Leishmania* species. Therefore, a comparative study was conducted of nucleobase transport in the amastigote forms of *L. mexicana*, as *L. major* amastigotes cannot, as yet, be cultured *in vitro*. Our results confirmed that hypoxanthine and allopurinol are taken up by a single plasma membrane transporter, LmexNBT1, characterized its substrate profile in detail, and proved it to be extremely similar to LmajNBT1. The finding that there is no significant difference in nucleobase

salvage in amastigotes and promastigotes contrasts with the salvage of nucleosides in these cells.

Nor is nucleobase transport identical in insect and mammalian stages of the related kinetoplastid *T. b. brucei*. It has been known for some time that bloodstream forms express two nucleobase transporters, H2 and H3 (De Koning and Jarvis, 1997b), whereas procyclic forms express only one, designated H1 (De Koning and Jarvis, 1997a), while expressing a second, higher affinity transporter as a response to purine starvation (De Koning, *et al.*, 2000b). We have now characterised this latter transporter, H4, and identified it as the gene product of the TbNBT1 gene (Chapter five) that we recently cloned and expressed in yeast as well as in *Xenopus* oocytes (Burchmore, *et al.*, 2003). We thus not only cloned the first protozoan nucleobase transporter, but also identified its physiological function in the parasite. Since the publication of our findings, the cloning of two more, closely related, nucleobase transporters from *T. b. brucei* (Henriques, *et al.*, 2003) and *L. major* (Sanchez, *et al.*, 2004b) have been reported. These further studies confirmed that, in protozoa, purine nucleobase transporters are encoded by ENT family genes (De Koning, *et al.*, 2005). However, it is as yet unclear whether pyrimidine transporters might be encoded by the same family. Given that the genome sequences of most major protozoan pathogens have now been published (Gardner, *et al.*, 2002; Berriman, *et al.*, 2005; Ivens, *et al.*, 2005), that most ENT genes have now been cloned and characterised (Landfear, *et al.*, 2004; De Koning, *et al.*, 2005; This thesis) and that multiple pyrimidine transport activities have been identified (De Koning and Jarvis, 1998; Papageourgiou, *et al.*, 2005; Gudin, *et al.*, submitted), it must be postulated that these transporters are encoded by an as yet unknown gene family.

Understanding the purine transporters and their unique substrate specificity might be exploited to deliver cytotoxic purine analogues selectively into the parasite but not human cells. The success of purine antimetabolites as therapeutic agents against some

infectious diseases and malignancies relies on the efficient accumulation and selective action on the target cell or organism. Therefore, a comprehensive study was conducted in our group to compare the transport of purine nucleobase analogues by the H2 nucleobase transporter of bloodstream form *Trypanosoma brucei brucei* and the human facilitative diffusion nucleobase transporter hFNT1 (Wallace, *et al.*, 2002; Wallace, *et al.*, 2004). This study was able to construct a model for the substrate recognition motif of both carriers, and demonstrated that selective uptake of purine antimetabolites by the TbH2, but not by hFNT1, is a viable option for the design of rational chemotherapy (Wallace, *et al.*, 2002).

Our approach of establishing substrate recognition motifs for nutrient transporters has recently been adopted for the study of human concentrative nucleoside transporters (hCNTs) and human equilibrative nucleoside transporters (hENTs) (see Chapter one), and the results seemed to confirm the possibility of selective accumulation by protozoa. The differences in permeant selectivities among hCNT1, hCNT2, and hCNT3 are also reflected in their different abilities to transport anticancer and antiviral nucleoside drugs (Zhang, *et al.*, 2003). The group of Cass has been investigating the interactions of uridine and its analogues, which are potential compounds in anticancer therapy, with hCNTs expressed in a yeast strain that lacks NT activity. However, it has been found that while hCNT1 and hCNT3 were very similar, the differences in their ligand recognition profiles were the result of differences in their permeant binding sites (Zhang, *et al.*, 2003).Using an improved yeast expression system to test uridine binding with hCNT1, hCNT2, and hCNT3, they identified the basis for differences in the ligand recognition and permeant selectivities (Zhang, *et al.*, 2005). Prior to that, a similar study by the same group also compared the interaction of uridine, cytidine, and other pyrimidine nucleoside analogues with the recombinant human equilibrative nucleoside transporters hENT1 and hENT2, which transports nucleobase, produced in yeast (Vickers, *et al.*, 2002; Vickers, *et al.*, 2004).

In our project, substrate specificities and affinities and recognition motifs were determined using data profiles obtained from the standard kinetics parameters. This allowed us to predict models of transporter-substrate interactions, which can help in selection or development of drugs that will be efficiently accumulated by a target cell or organelle. For example, a new class of tricyclic purine antimetabolites was tested for toxicity in our laboratory and displayed a remarkable selectivity for the parasite's transporter (up to 1000-fold higher affinity than for the corresponding human transporter) and some of them showed greater trypanocidal activity than existing hypoxanthine or guanine antimetabolites *in vitro* (Wallace, *et al*, 2004).

Our results from the investigation of the transporters in the apicomplexan protozoan *Toxoplasma gondii*, allowed us to predict a model of interaction with the pyrimidine nucleosides, such as uridine, with TgAT2. We have demonstrated that this carrier efficiently transports pyrimidine nucleosides but not nucleobases. The crucial interactions that allow transport of pyrimidines in addition to purines appeared to consist of strong hydrogen bonds to the 3' and 5' hydroxyl groups in the ribose ring, and through a hydrogen bond at N(3)H in the base ring. Therefore, the binding architecture of this transporter, with pyrimidine nucleosides, is very similar to interactions between these substrates with the human concentrative nucleoside transporters (hCNTs) and human equilibrative nucleoside transporters (hENTs) (Vickers, *et al.*, 2002; Zhang, *et al.*, 2003; Vickers, *et al.*, 2004; Zhang, *et al.*, 2005). These models can predict that the same therapeutic nucleosides efficiently transported by hCNT3 (Ritzel, *et al.*, 2001a; Ritzel, *et al.*, 2001b), including gemcitabine, 5-fluoro-2'-deoxyuridine, fludarabine, and cladrabine will be excellent permeants for TgAT2. This makes TgAT2 a very promising target for therapeutic agents, as these compounds would need to cross the host cell membrane in order to get in to these parasites.

The current study also clearly shows that the substrate recognition models for the nucleobase transporters of *L. major* (LmajNBT1), *L. mexicana* (LmexNBT1), and *T.*

brucei (H2) are almost identical. For instance, these transporters appear to make similar hydrogen bonds with hypoxanthine, through N(1)H, N3, N7 and N(9)H, but this common motif is very different from that of the human facilitative diffusion nucleobase transporter hFNT1 (Wallace, *et al.*, 2002; Chapter three). This suggests that these related kinetoplastids would accumulate similar purine antimetabolites, which could be designed to be excluded from human cells. Therefore, any ideal compound that should be synthesised has to be compatible with the models proposed. Looking at the binding sites of the transporter pocket, amino acid side chains can form a variety of hydrogen bonds, and in fact, eleven of the 21 amino acids can form hydrogen bonds through their side chains. Free amino group can serve as H-bond donors and free carboxyl groups can serve as hydrogen bond acceptors. Figure 8.1 shows, for example, a predicted model of interaction between the purine analogue 6-mercaptopurine and binding sites of the protozoan nucleobase transporter and the human nucleobase transporter hFNT1. 6-Mercaptopurine is a successful agent for treatment of cancer (Galmarini, *et al.*, 2002). The cytotoxicity of this class of purines is thought to depend mainly on the incorporation of their phosphorylated derivates into DNA, which interferes with the function of DNA polymerases, ligases, and endonucleases. Moreover, thiopurines may also cause toxic effects by inhibiting other enzymes such as 5-phosphoribosyl-l-pyrophosphate amidotransferase, IMP dehydrogenase, or ribonucleotide reductase, which are all involved in *de novo* purine synthesis enzymes. This might explain the failure of this compound against protozoan parasites, as these parasites cannot synthesis purines *de novo*.

Preliminary toxicity studies on human cell lines, with selected purine analogues that show sub-micromolar *in vitro* antiprotozoal activities, are currently in progress in our group. Some of these purine analogues have been rationally designed for selective uptake by the parasite, and measurements of the affinities for human and parasite transporters have indeed shown higher affinity for the protozoan transporters (Rodenko and De Koning, unpublished). With our understanding of purine uptake by

some of the major protozoan parasites now virtually complete, the outlook for development of selective purine-based drugs appears highly promising.

After understanding the first step of purine salvage in these parasites, the conversion of any promising compound by the parasite's metabolism to a form harmful to the organism is crucial. Selective toxicity therefore needs to be the result of either selective uptake or differences in the metabolic enzymes for pathways. Therapeutic action will then depend on such enzymes as the phosphoribosyltransferases, among others, that convert the analogues into nucleotides, though some purines, including kinase inhibitors, may not need any metabolic conversion for their mode of action. Combining our knowledge of the purine transporters with a good understanding of the metabolism of different purine analogues, would ensure selective and efficient salvage of such designer drugs.

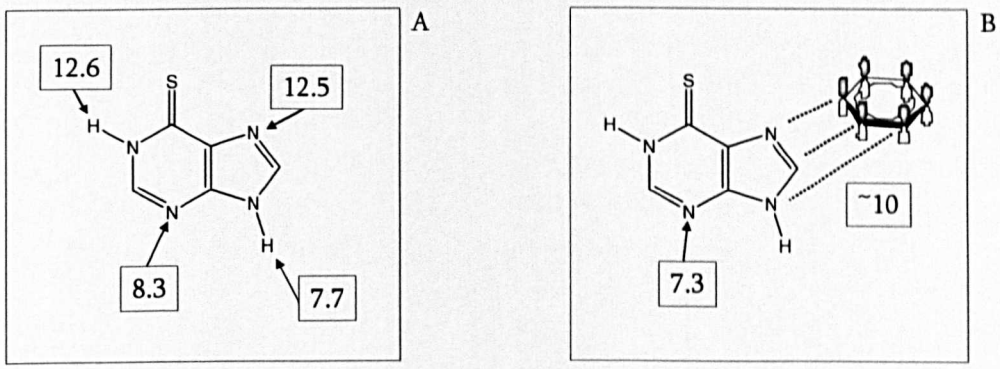


FIGURE 8.1 Model for the interactions between the TbH2 (A) and hFNT1 (B) transporters and purine analogue 6-mercaptopurine. Estimated Gibbs free energy for proposed bonds are indicated as – kJ/mol. This model was adapted from Wallace et al. (2002).

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APPENDICES

Appendix I: Media, Buffers, Solutions

HOMEM MEDIUM

Components	Quantity per litre
S-MEM (Gibco)	1 g
Glucose	2 g
Sodium bicarbonate	0.3 g
Sodium pyruvate	0.11 g
p-aminobenzoic acid	1 mg
Biotin	0.1 mg
HEPES	5.96 g
MEM Amino acids (50X)	10 ml
MEM Non-essential Amino acids (100X) (Gibco)	10 ml

Make up to pH 7.5-7.6 as the pH will, after filter sterilising, drop to 7.0-7.2. Filter sterilise using 0.22 µm Millipore filter into sterile bottles. Complete medium (for *L. major*) contains 10% Fetal Calf Serum (FCS) and 90% HOMEM. Store at 4 °C to use within 12 weeks and medium appears to be stable at -20 °C for at least 6 months.

HMI-9 MEDIUM

Components	Quantity per litre
Iscoves MEM	730 ml
Hypoxanthine (or adenosine for purine nucleobase-free)	2 mM
Kanamycine	30 µg/ml
Penicillin	50 u/ml
Streptomycin	50 µg/ml
Bathocuprione disulphonic acid	50 µM
Thymidine	160 µM
Pyruvate	1 mM

β-mercaptoethanol	2 mM
L-cysteine	1 mM
$NaHCO_3$	3.02 g

Adjust to final pH 7.5 and filter sterilise. Add 20% of Fetal Calf Serum (FCS) or dialysed FCS for purine nucleobase-free.

SCHNEIDER'S *DROSOPHILA* MEDIUM

Components	Quantity per litre
Calcium Chloride (anhydrous	0.6 g
Magnesium Sulfate	1.8 g
Potassium Chloride	1.6 g
Potassium Phosphate Monobasic	0.45 g
Sodium Bicarbonate	0.4 g
Sodium Chloride	2.1 g
Sodium Phosphate Dibasic	0.7 g
B- alanine	0.5 g
L-arginine	0.6 g
L-aspartic acid	0.4 g
L-cystine.2HCl	0.02 g
L-cysteine	0.06 g
L-glutamic acid	0.8 g
L-glutamine	1.8 g
Glycine	0.25 g
L-histidine	0.4 g
L-isoleucine	0.15 g
L-leucine	0.15 g
L-lysine	1.65 g
L-methionine	0.15 g
L-proline	1.7 g
L-serine	0.25 g
L-threonine	0.35 g
L-tryptophan	0.1 g
L-tyrosine.2Na.2H2O	0.72 g
L-valine	0.3 g
Fumaric acid	0.06 g
Glucose	2 g
α-ketoglutaric acid	0.35 g
Malic acid	0.6 g
Succinic acid	0.06 g

Trehalose	2 g
Yeast extract	2 g

The pH of the medium was adjusted to 5.5 with 1.0 M HCl. Filter sterilise and add 0.3% gentamicin. The medium was supplemented with 20% of Fetal Calf Serum (FCS).

SDM-79 MEDIUM

Components	Quantity per litre
S-MEM F14 powder	7 g
Medium 199 TC45 powder	2 g
MEM amino acids (50X)	8 ml
MEM Non-essential Amino acids (100X)	6 ml
Glucose	1 g
HEPES	8 g
MOPS	5 g
$NaHCO_3$	2 g
Sodium pyruvate	100 mg
L-alanine	200 mg
L-arginine	100 mg
L-glutamine	300 mg
L-methionine	70 mg
L-phenylalanine	80 mg
L-proline	600 mg
L-serine	60 mg
L-taurine	160 mg
L-threonine	359 mg
L-tyrosine	100 mg
Adenosine	10 mg
Guanosine	10 mg
Glucosamine HCl	50 mg
Folic acid	4 mg
p-aminobenzoic acid	2 mg
Biotin	0.2 mg

Adjust to final pH 7.3 and filter sterilise. Add 10% of Fetal Calf Serum (FCS). Store at 4 °C to use within 12 weeks

PURINE-FREE TRYPANOSOME MEDIUM (PFTM)

Components	Quantity per litre
RPMI 1640 medium	130 ml
S-MEM powder	7 g
MEM (50X) amino acids	8 ml
MEM Non-essential Amino acids (100X)	6 ml
$CaCl_2.2H_2O$	39 mg
Glucose	1 g
HEPES	8 g
MOPS	5 g
$NaHCO_3$	2 g
Sodium pyruvate	0.2 g
L-alanine	0.2 g
L-arginine	0.1 g
L-glutamine	0.3 g
L-methionine	70 mg
L-phenylalanine	80 mg
L-proline	0.6 g
L-serine	60 mg
L-threonine	0.35 g
L-tyrosine	0.1 g
L-taurine	0.3 g
Glucosamine HCl	50 mg
Folic acid	5 mg
p-aminobenzoic acid	20 mg
Biotin	0.2 mg
Sodium acetate	6.5 mg

Adjust to final pH 7.3 and filter sterilise. Supplement with 7.5 mg/l bovine hemin, 5% dialysed Fetal Calf Serum (FCS) and 0.2 mM final of inosine.

YPAD (YEAST EXTRACT-PEPTONE-DEXTROSE AND ADENINE MEDIUM)

Components	Quantities
Yeast extract	6 g
Peptone	12 g
Glucose	12 g
Adenine hemisulphate	60 mg

Bacto-agar (if solid medium required)	10 g
dH_2O	600 ml

Adjust to final pH 6.0, autoclave at 121 °C for 15 minutes and store at 4 °C.

SC-URA (SYNTHETIC COMPLETE MEDIUM MINUS URACIL)

Components		Quantities
Difco Yeast Nitrogen Base without amino acids		4 g
Glucose		12 g
Synthetic complete drop out mix:		0.5 g
Adenine hemisulphate	2 g	
Arginine HCl	2 g	
Histidine HCL	2 g	
Isoleucine	2 g	
Leucine	4 g	
Lysine HCl	2 g	
Methionine	2 g	
Phenylalanine	3 g	
Homoserine	6 g	
Tryptophan	3 g	
Tyrosine	2 g	
Valine	9 g	
Bacto-agar (if solid medium required)		10 g
dH2O		600 ml

Add all ingredients (except agar) to water and adjust pH to 5.6 with 10 M NaOH. NOTE- this step is important for highest efficiency transformation. Autoclave at 121 °C for 15 minutes.

CBSS BUFFER (CARTER'S BALANCED SALT SOLUTION)

Components	Quantity per litre
MES	6.4 g
NaCl	5.7 g
KCl	342.9 mg
CaCl2	44.1 mg

MgSO4	17.2 mg
NaH2PO4	904.8 mg
MgCl2	60.9 mg
Glucose	2.7 g

Adjust to final pH 6.0 and store at 4°C.

ASSAY BUFFER

Components	Quantity per litre
Glucose	2.53 g
HEPES	8.0 g
MOPS	5.0 g
$NaHCO_3$	2.0 g
KCL	347.5 mg
$MgCl.6H_2O$	62.5 mg
NaCl	5.7 g
$NaH2PO_4.2H_2O$	913.5 mg
$CaCl_2.2H_2O$	40.7 mg
$MgSO_4.7H_2O$	19.9 mg

Adjust to final pH 7.3 and store at 4°C. Note- for uptake assays using yeast, assay buffer without the addition of glucose is used for the final wash and re-suspension (all other quantities remain the same).

OIL MIXTURE

Components	Quantities
Mineral oil (Sigma)	50 ml
di-n-butyl phthalate	350 ml

2% SDS

Components	Quantities
Sodium dodecly sulphate	10 g
dH_2O	500 ml

PSG (PHOSPHATE-BUFFERED SALINE PLUS GLUCOSE)

Components	Quantity per litre
Na2HPO4 (anhydrous)	13.48 g
NaH2PO4.2H2O	0.78 g
NaCl	4.25 g

Make to one litre with dH2O (PS buffer). Dissolve 10 g of glucose in approximately 200 ml of dH2O (Glucose solution). Add six volumes of PS to four volume of glucose solution (PSG buffer). Adjust to pH 8.0 exactly and store at 4°C.

DNA EXTRACTION BUFFER

Components	Quantities
(1.0 M) Tris (pH 8)	10 ml
(0.5 M) EDTA	2 ml
(5.0 M) NaCl	4 ml
dH_2O	to 200 ml

NAD DIAPHORASE ASSAY REACTION BUFFER

Components	Quantities
NADH-disodium salt	4 mg
0.1 M Phosphate buffer pH 7.3	0.8 ml
dH_2O	1.2 ml

TBE RUNNING BUFFER

Components	Quantities
Tris (pH 8)	53.9 g
Boric acid	27.5 g
EDTA	2.3 g
dH_2O	to 5 litres

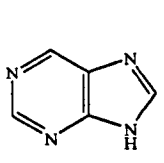
LB MEDIUM

Components	Quantity per litre
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

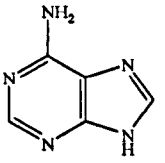
Adjust to final pH 7.0, autoclave at 121 °C for 15 minutes and store at 4 °C.

Appendix II: Supplementary Data

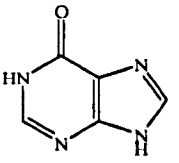
A. Structure of some purine nucleobases and nucleosides and their analogues used to construct the models for the interactions between the transporters binding sites and the permeants.



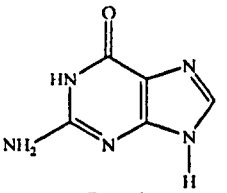
Purine



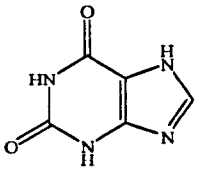
adenine



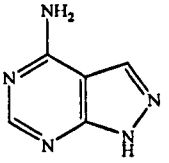
Hypoxanthine



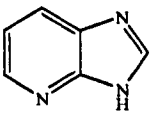
Guanine



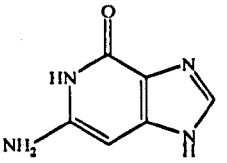
Xanthine



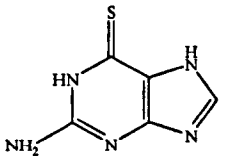
Allopurinol



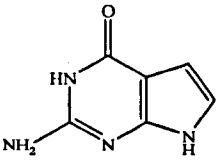
1-Deazapurine



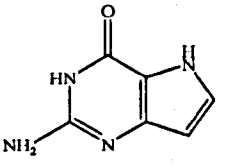
3-Deazaguanine



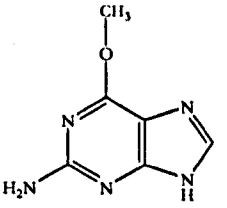
6-Thioguanine



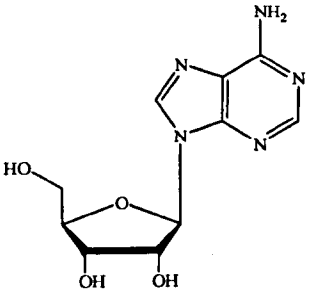
7-Deazaguanine



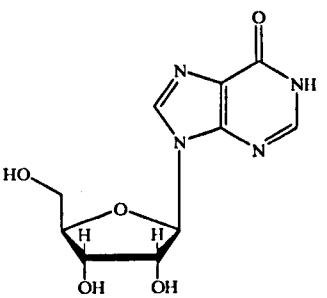
9-Deazaguanine



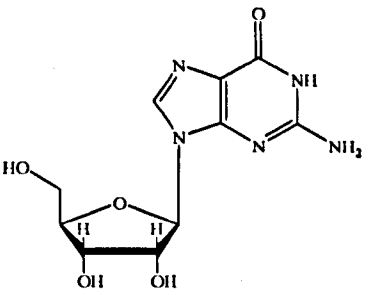
6-methylguanine



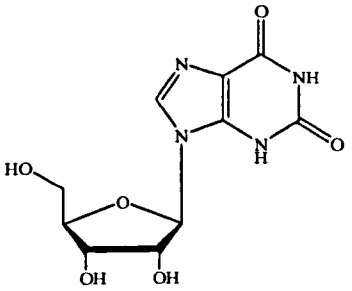
Adenosine



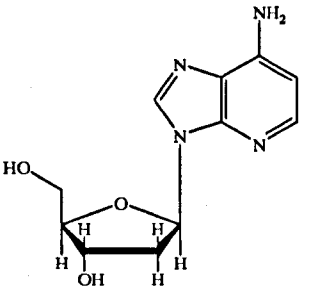
Inosine



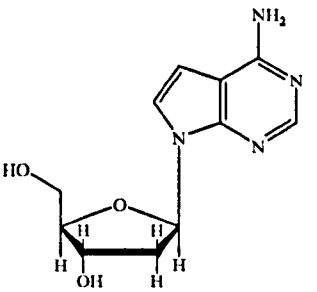
Guanosine



Xanthosine

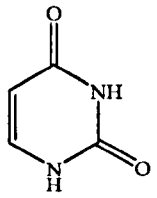


1-Deazaadenosine

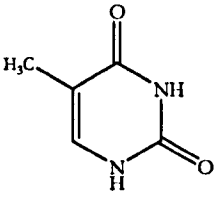


7-Deazaadenosine

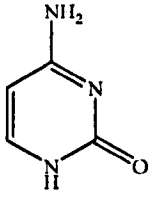
B. Structure of some pyrimidine nucleobases and nucleosides and their analogues used to construct the models for the interactions between the transporters binding sites and the permeants.



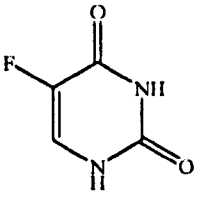
Uracil



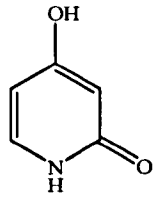
Thymine



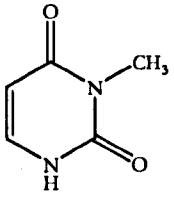
Cytosine



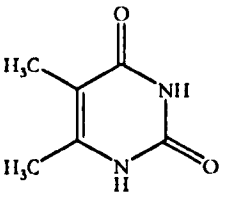
5-Fluorouracil



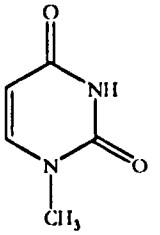
3-Deazauracil



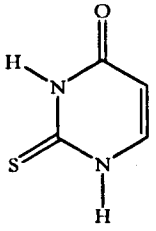
3-Methyluracil



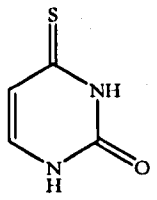
5,6-Dimethyluracil



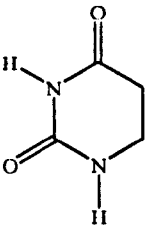
1-Methyluracil



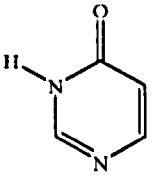
Thiouracil



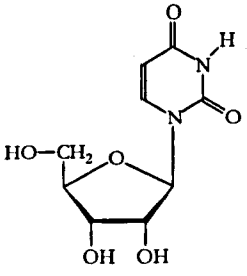
4-Thiouracil



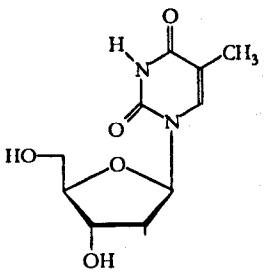
5,6-Dihydrouracil



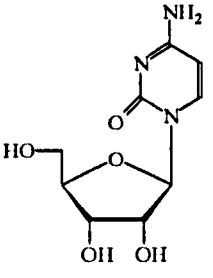
(3H)-Pyrimidone



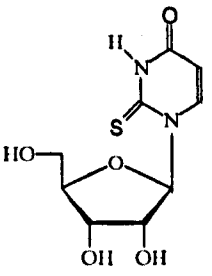
Uridine



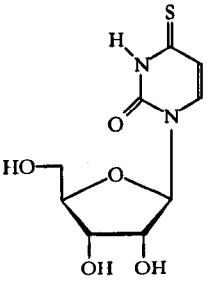
Thymidine



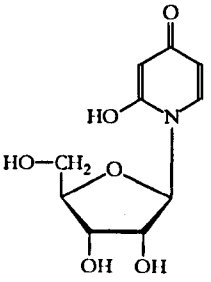
Cytidine



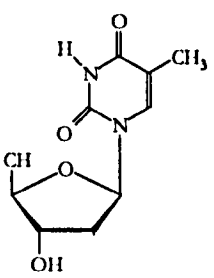
2-Thiouridine



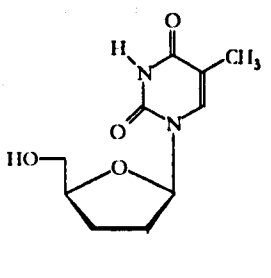
4-Thiouridine



3-Deazauridine



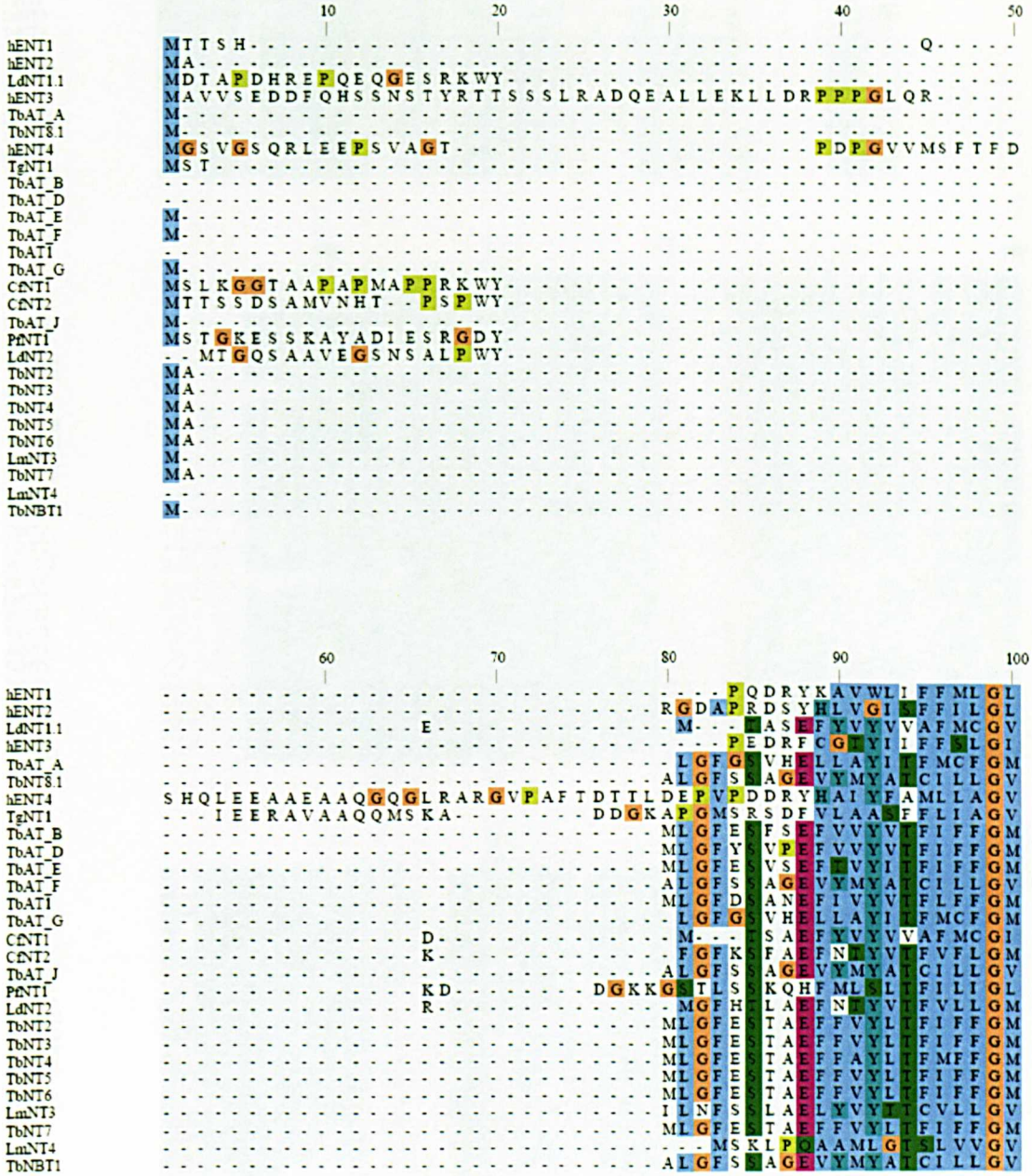
5'-Deoxythymidine

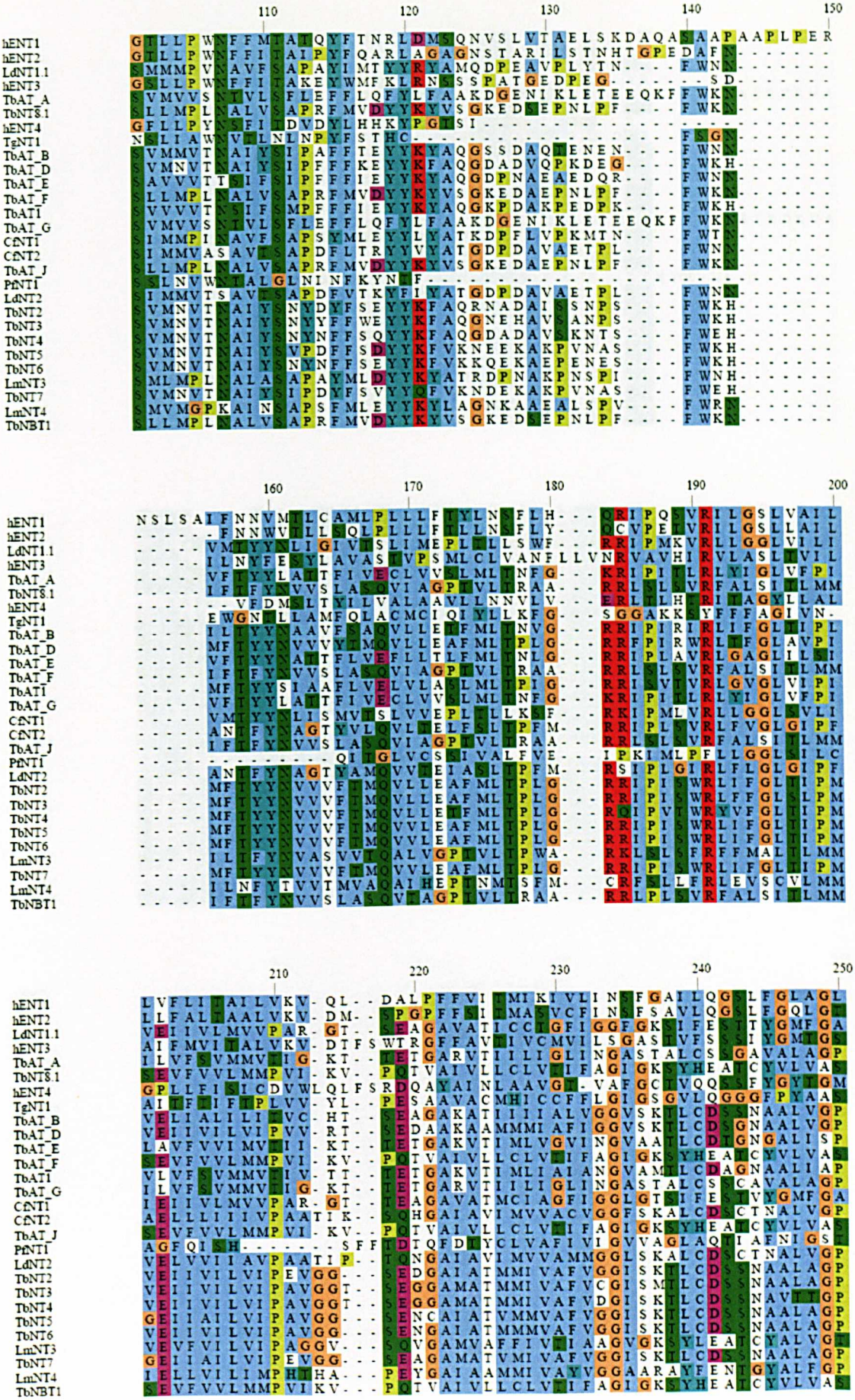


3'-Deoxythymidine

C. Multiple alignment of human and protozoan ENT genes. Alignment was generated using the program dialign (Morgenstern, B. (1999) DIALIGN 2: improvement of the segment-to-segment approach to multiple sequence alignment. Bioinformatics 15, 211–218) using the blosum62 matrix. Reproduced from De koning et al. (2005).

Key. aliphatic, aromatic/hydrophobic, blue; positive, red; negative, magenta; hydrophilic, green; praline, yellow; glycine, orange.

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TgNT1
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TbAT_D
TbAT_E
TbAT_F
TbAT1
TbAT_G
CfNT1
CfNT2
TbAT_J
PfNT1
LdNT2
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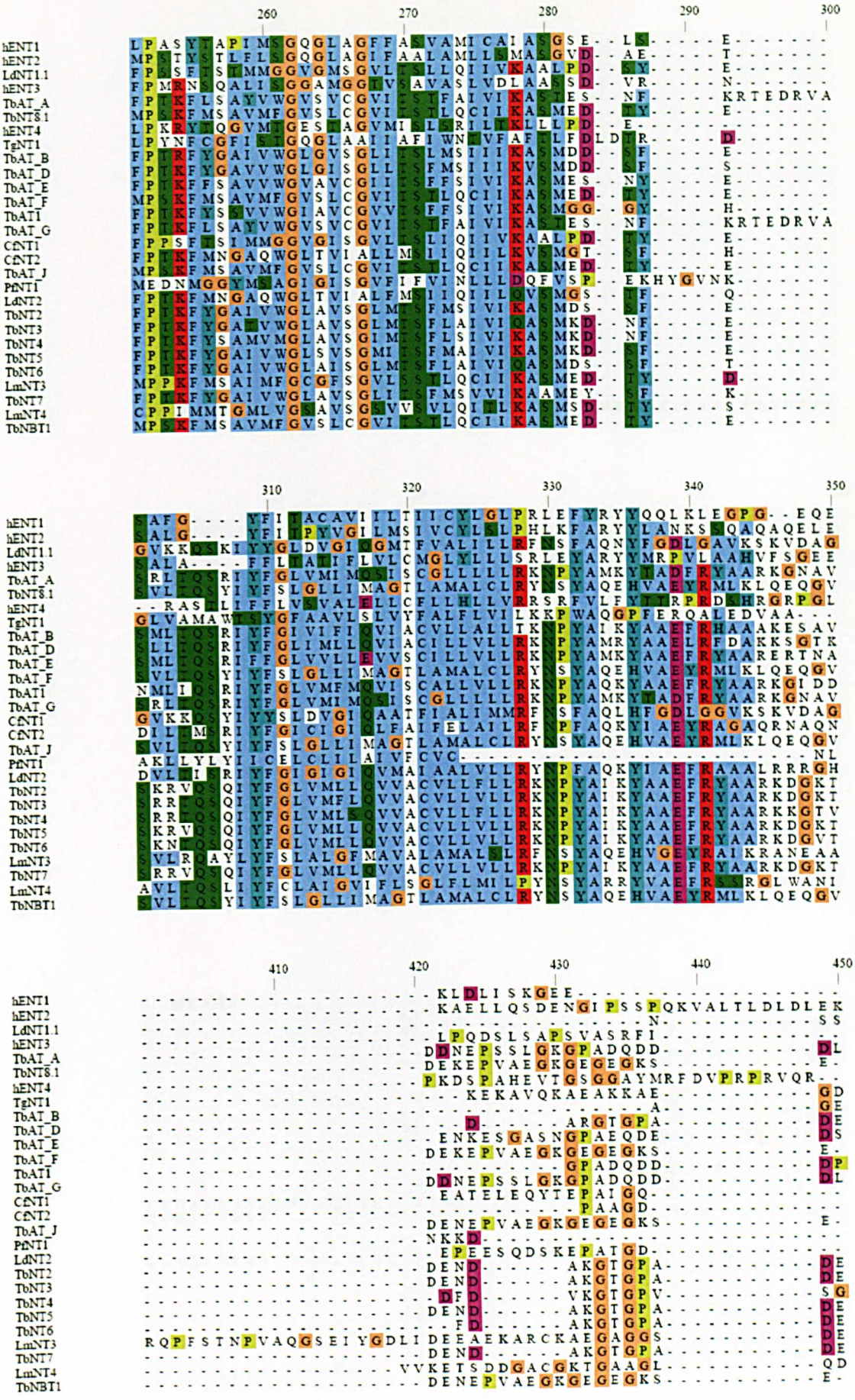
TbNT7
LmNT4
TbNBT1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hENT4
TgNT1
TbAT_B
TbAT_D
TbAT_E
TbAT_F
TbAT1
TbAT_G
CfNT1
CfNT2
TbAT_J
PfNT1
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TbNT7
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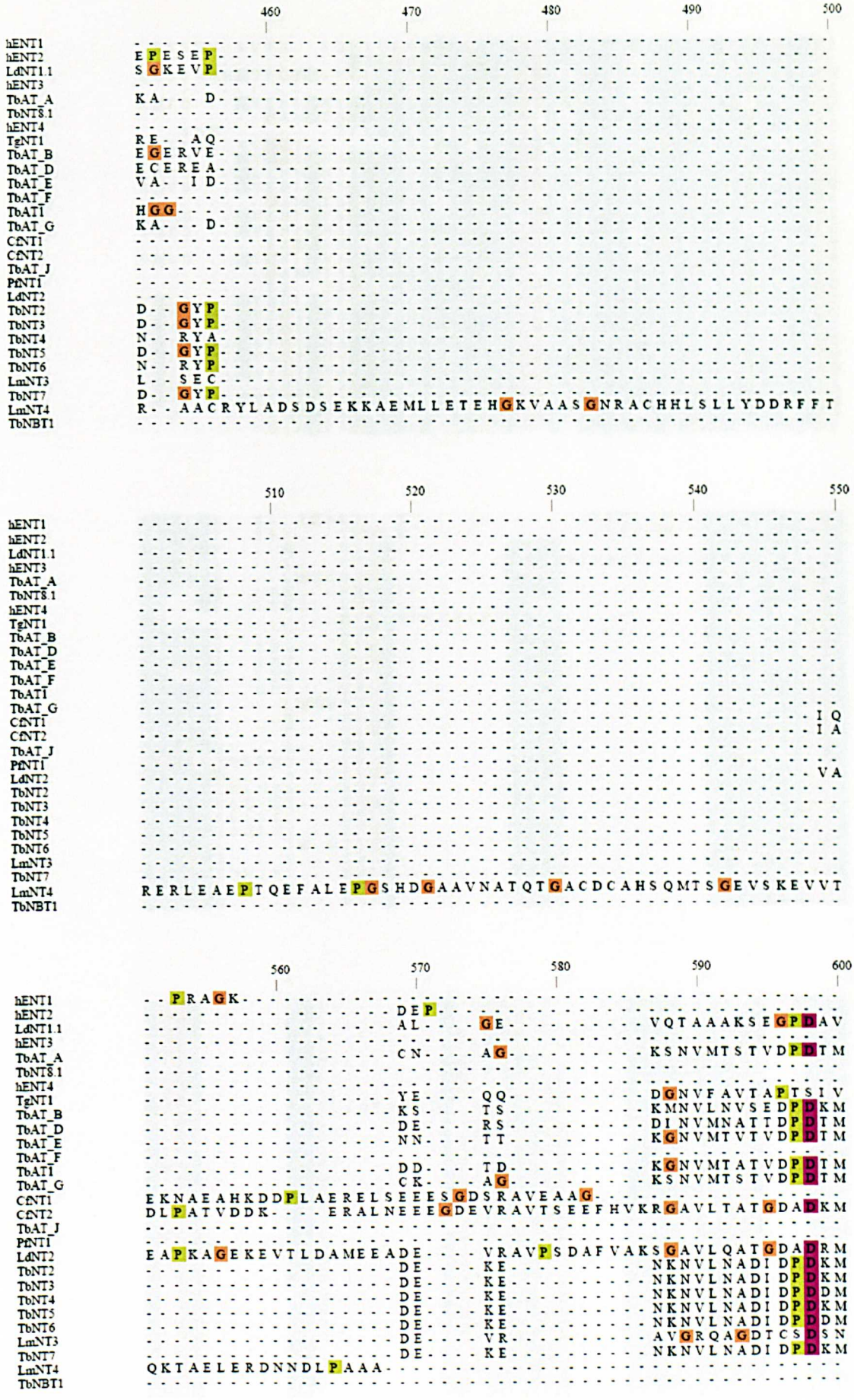
TbNBT1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TbNT6
LmNT3
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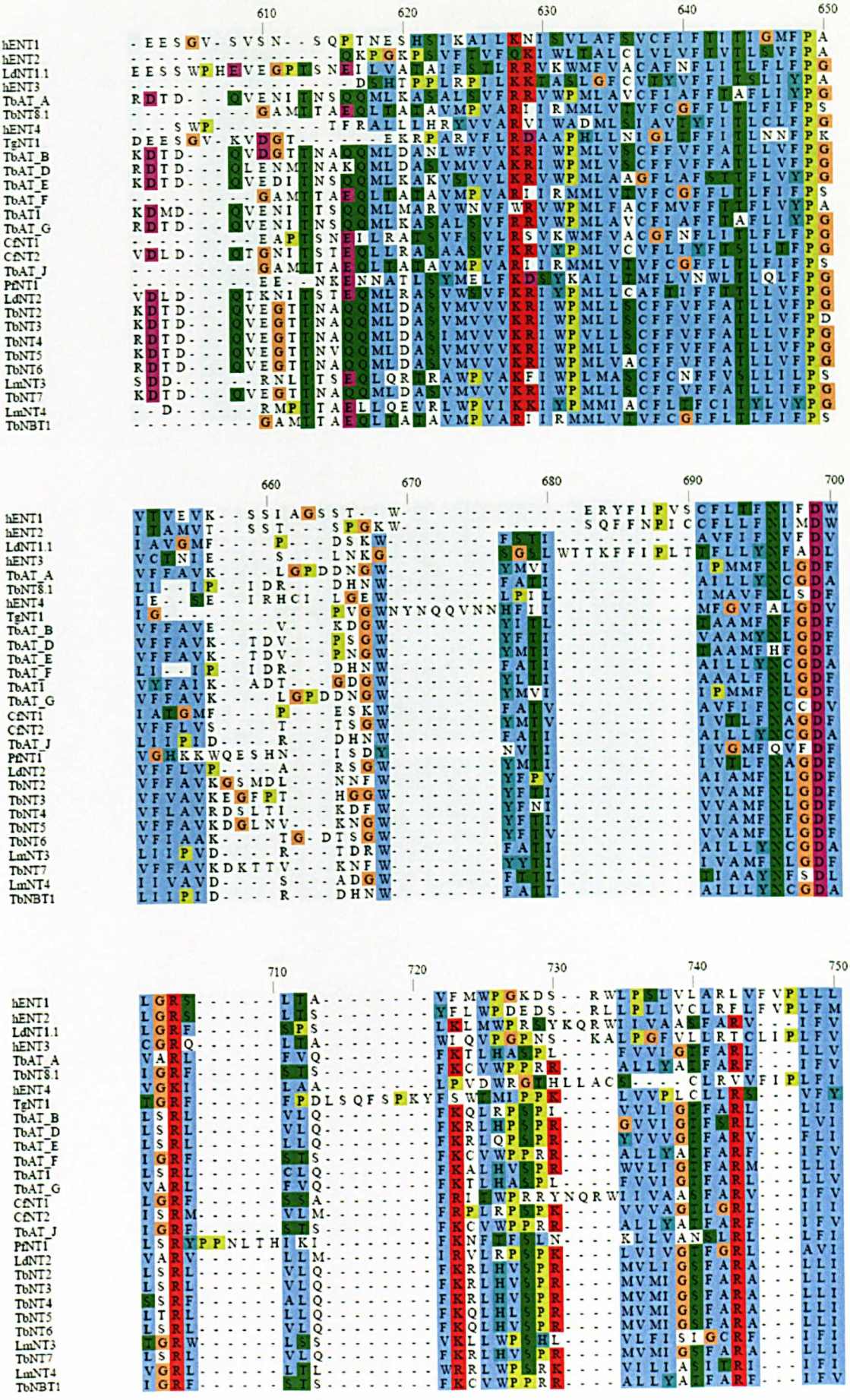
LmNT4
TbNBT1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620
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650
660
670
680
690
700
710
720
730
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hENT2
LdNT1.1
hENT3
TbAT_A
TbNT8.1
hENT4
TgNT1
TbAT_B
TbAT_D
TbAT_E
TbAT_F
TbAT1
TbAT_G
CfNT1
CfNT2
TbAT_J
PfNT1
LdNT2
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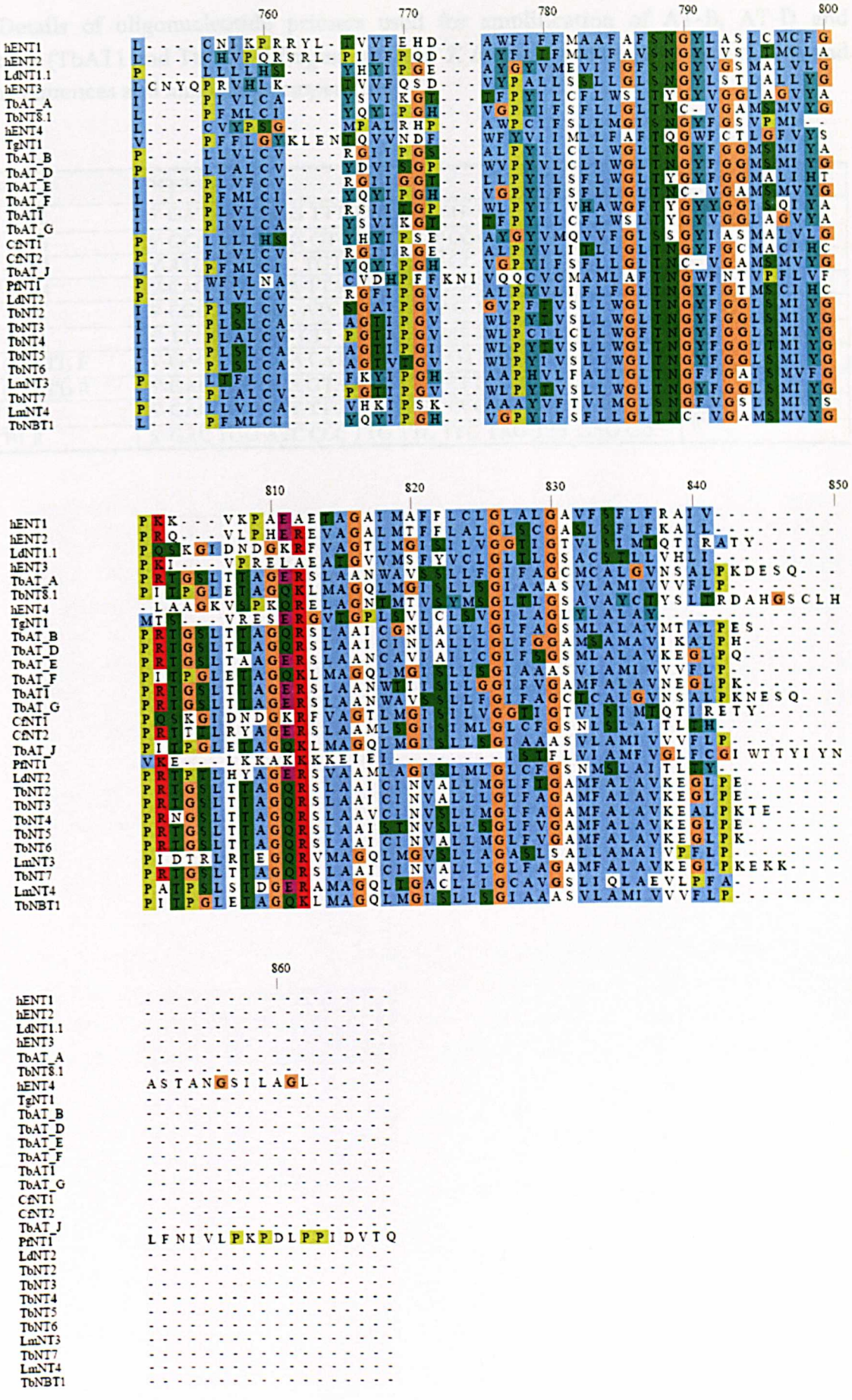
TbNT4
TbNT5
TbNT6
LmNT3
TbNT7
LmNT4
TbNBT1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TbAT_B
TbAT_D
TbAT_E
TbAT_F
TbATI
TbAT_G
CfNT1
CfNT2
TbAT_J
PfNT1
LdNT2
TbNT2
TbNT3
TbNT4
TbNT5
TbNT6
LmNT3
TbNT7
LmNT4
TbNBT1
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D. Details of oligonucleotide primers used for amplification of AT-B, AT-D and controls (TbAT1 and TbNBT1) fragments from *T. brucei* genomic DNA and cDNA, and their sequences and annealing temperature.

Primer	Sequence	Annealing temp
AT-D F2	5'-GAT GCT CGG TTT TTA TTC AG-3'	52 °C
AT-D F3	5'-GCA AGT AAG GTC GAT GCT CGG-3'	"
AT-D R2	5'-ATG TCA CAC TGT TAT TTC AGC-3'	"
AT-D R4	5'-CAG CTG TAA TTA ATG CGG AAG AG-3'	"
AT-B F1	5'-CGC TTC ACC ATC TAG CTG AG-3'	56 °C
AT-B R1	5'-TTA CCT CCT CTT AGG GAC AG-3'	"
P2 (TbAT1) F	5'-GAC TGT CGA CAT GCT CGG GTT TGA CTC AGC C-3'	55 °C
P2 (TbAT1) R	5'-GAC TCT GCA GTA GTG CTA CTT GGG AAG CCC-3'	"
NTB1 F	5'-GAC TAA GCT TTG GAG TGT CCC TTC TAA TGC C-3'	55 °C
NTB1 R	5'-GAC TGG ATC CCC TTG TTC TTG TAG TTT GAG C-3'	"

Appendix III: Publications

1. Al Salabi, M. I., & De Koning, H. P. (2005) Novel ENT nucleoside transporters in *Trypanosoma brucei*; cloning of additional genes and comparison of substrate-transporter interactions. In preparation.

2. Al Salabi, M. I., & De Koning, H. P. (2005) Purine nucleobase transport in amastigotes of *Leishmania mexicana*: involvement in allopurinol uptake. *Antimicrob Agents Chemother.* 49:3682-9.

3. Al Salabi, M. I., Wallace, L. J., & De Koning H. P. (2003) A *Leishmania major* nucleobase transporter responsible for allopurinol uptake is a functional homolog of the *Trypanosoma brucei* H2 transporter. *Mol Pharmacol.* 63:814-820.

4. Burchmore, R. J., Wallace, L. J., Candlish, D., Al Salabi, M. I., Beal, P. R., Barrett, M. P., Baldwin, S. A., & De Koning H. P. (2003) Cloning, heterologous expression, and in situ characterization of the first high affinity nucleobase transporter from a protozoan. *J Biol Chem.* 278:23502-23507.

5. De Koning, H. P., Al Salabi, M. I., Cohen, A. M., Coombs, G. H., & Wastling, J. M. (2003) Identification and characterisation of high affinity nucleoside and nucleobase transporters in *Toxoplasma gondii*. *Int J Parasitol.* 33:821-831.

6. Natto, M. J., Wallace, L. J., Candlish, D., Al Salabi M. I., Coutts, S. E., & De Koning H. P. (2005) *Trypanosoma brucei*: expression of multiple purine transporters prevents the development of allopurinol resistance. *Exp Parasitol.* 109:80-86.

7. Papageorgiou, I. G., Yakob, L., Al Salabi, M. I., Diallinas, G., Soteriadou, K. P., & De Koning H. P. (2005) Identification of the first pyrimidine nucleobase transporter in *Leishmania*: similarities with the *Trypanosoma brucei* U1 transporter and antileishmanial activity of uracil analogues. *Parasitol.* 130:1-9.