INVESTIGATIONS ON MECHANISMS OF ACQUISITION OF DRUG RESISTANCE IN TRYPANOSOMES

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A thesis submitted for the degree of Doctor of Philosophy

The University of Edinburgh

1997

DEDICATION

To the Lord Most Worthy; Cephas, Saanyol and Sena; mum, Mrs Rachael Ngizan Ityondo & dad, Mr. D.P. Ityondo; and entire family; and our late mum, Mrs Ashiewua Suswam; for their inspiration, love and admiration.

DECLARATION

The study presented in this thesis is the result of work carried out by me, unless where otherwise acknowledged. No part of this work has been submitted elsewhere for any Degree, Diploma, or other qualifications.

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ACKNOWLEDGEMENTS

SUMMARY

The widespread occurrence of drug resistant trypanosomes has constituted a major hindrance to the control of animal trypanosomosis. The study described here was an investigation into the mechanisms of acquisition of drug resistance by *Trypanosoma evansi*. The main aim was to determine the biochemical basis for the development of resistance to the melaminophenyl arsenical drugs, mel Cy and mel B. Experiments were designed to compare the mode of development of resistance *in vitro* with *in vivo*-derived resistance and to determine the effect of host immunity on resistance development. Resistance to mel Cy was induced in a sensitive stock of *T. evansi* by infection and treatment with sub-curative levels of drug, in immunosuppressed and immunocompetent mice. Induction was also carried out *in vitro* by cultivation of the culture-adapted *T. evansi* isolate in the continuous presence of increasing subinhibitory concentrations of mel Cy. Resistance was measured using *in vivo* drug sensitivity tests and, *in vitro* growth inhibition assays.

High levels (up to 300-fold) of resistance were attained within 6 months of induction in immunosuppressed mice and *in vitro*. It was not possible to produce resistance in immunocompetent mice, even after 24 months. *In vivo* drug resistant trypanosomes maintained their infectivity but virulence was reduced. Resistance induced by mel Cy was characterised with respect to stability of resistance and cross resistance to other trypanocides. The resistance developed from either immunosuppressed mice or *in vitro* continued to be expressed in immunocompetent mice treated with the trypanocidal drug. After withdrawal of drug *in vitro*-induced resistance remained stable whereas *in vivo*-derived resistance was reduced by over 50%. Mel Cy-induced resistance conferred cross resistance to other trypanocidal drugs, namely, mel B, Berenil®and, to a limited extent, quinapyramine, but not suramin. Sub-populations of trypanosomes tolerant to the selection dose (20 ηg/ml) of mel Cy were not found in the unselected parent clone. Increased sensitivity to

quinapyramine and suramin was observed after adaptation of the drug sensitive isolate to *in vitro* culture.

Uptake of melaminophenyl arsenicals into *T. evansi* was investigated using an *in vitro* lysis assay. Mel Cy and mel B caused lysis of drug sensitive and drug resistant trypanosomes in a dose-dependent and saturable manner. Mel B was less effective in lysis of drug sensitive trypanosomes but very potent in lysis of drug resistant parasites. The *in vitro* lysis assay differentiated between drug sensitive and drug resistant trypanosomes. Mel Cy lysis was blocked by adenosine, adenine and Berenil®, but not inosine. Mel B lysis was inhibited by Berenil® but not adenosine, adenine or inosine. These results indicate that mel Cy competes for an adenine/adenosine transporter whereas mel B does not.

Uptake of adenosine into drug sensitive and drug resistant trypanosomes was investigated using competitive inhibition experiments. Adenine, inosine and Berenil® all inhibited adenosine uptake in a concentration-dependent and saturable manner. Percentage inhibitions in the drug sensitive parasites were 53.6, 23.2, and 33.2, respectively, for adenine, inosine and Berenil®. Combinations of adenine and inosine resulted in total inhibition of uptake of adenosine. These findings suggest that *T. evansi* possesses a bipartite P1/P2 adenosine transport system. Mel Cy was taken up by the P2 transporter. The P2 transporter was maintained in arsenical resistant *T. evansi*. However, uptake on the P2 was reduced 3-10 times in different resistant lines. Reduction in uptake at the P2 transporter resulted in reductions in total adenosine uptake by the resistant parasites. Moderate reductions in P1 were also observed. Berenil® also interacted with the P2 transporter in drug sensitive trypanosomes. Interaction of Berenil® with the P2 transporter was greatly reduced in the resistant population. Thus, the cross resistance that develops between melaminophenyl arsenicals and diamidines is a result of their common mechanisms of uptake.

Kinetic studies on the P1 and P2 adenosine transporters were carried out on both the drug sensitive and the drug resistant *T. evansi*. Uptake rates including A_{max} (maximum transport capacity), Rate constant (T), and initial rates of uptake,

were determined. In the drug sensitive trypanosomes the P2 transporter was a larger transport process. A_{max} for P2 was 2 times greater than A_{max} for P1 transporter. Rate constant and initial rates for P1 and P2 were similar. The A_{max} at the P2 was decreased 3.4 times in drug resistant trypanosomes. The Rate constant at both the P1 and P2 were increased. Initial rate at the P2 was decreased 2-fold. Reduced A_{max} accounted for the reduced uptake at the P2 transporter. Michaelis-Menten kinetic parameters were also determined for the P1 and P2 transporters. In drug sensitive trypanosomes the U_{max} at the P2 (28.9 $\rho mol/10^8 cells/5min.$) was 2-fold greater than the U_{max} (12.4 $\rho mol/10^8 cells/5min$) at the P1. The K_m for the P1 (0.07 μM) was 10-fold lower than the K_m for P2 (0.74 μM). The U_{max} (3.3 $\rho mol/10^8 cells/5min.$) for the P2 in drug resistant trypanosomes was reduced 9-fold. The K_m (0.1 μM) was decreased 7-fold. Kinetic changes for total adenosine uptake in trypanosomes were also determined. It was found that reduction in uptake capacity of P2 in arsenical resistant *T. evansi* was not due to decreased affinity. Rather a decrease in numbers of transporter molecules was responsible for the decrease in adenosine uptake at the P2. The changes in kinetic parameters of adenosine transport suggested that the P2 in drug resistant trypanosomes had greatly undergone mutation (P2R), as such it interacted with Berenil[®] in a different way.

The kinetics of inhibition of adenosine uptake showed that inosine, adenine and Berenil[®] inhibited uptake on either the P1 or P2 transporters with very low K_i values. In the drug sensitive trypanosomes K_i for inosine or adenine alone were 0.002 μM or 0.28 μM, respectively. K_i for Berenil[®] alone was 0.00 μM indicating that there was little or no interaction. On the P1 the K_i for inosine was 0.03 μM. There was no interaction with Berenil[®] on the P1. The K_i for adenine and Berenil[®] on the P2 were 0.3 and 0.01 μM, respectively. In the drug resistant line the K_i for inosine on P1 (0.06 μM) was similar to that of the drug sensitive line. However there was a 7-fold increase (1.94 μM) in K_i for adenine on the P2. Based on changes in all the kinetic parameters of what should be the P2 it was concluded that a new transporter (P3) was present in the arsenical resistant

trypanosomes. This transporter may have been selected for during induction of resistance to mel Cy, or may be an adaptation by the resistant trypanosomes following gross reductions in activity of the P2 transporter.

Molecular studies involving isolation and characterisation of the P2 and P3 transporters may fully elucidate the specific mutations responsible for arsenical resistance in trypanosomes. It will also be interesting to study the interaction of the P3 transporter with other alternative substrates, including toxic adenosine analogues. This may provide useful information for improved chemotherapy of trypanosomes resistant to arsenical compounds.

TABLE OF CONTENTS

ABBREVIATIONS

μg	Microgram
mg	Miligram
gm	Gram
kg	Kilogram
μM	Micromolar
μl	Microlitre
μm	Micrometre
ηM	Nanomolar
mM	Milimolar
ml	Mililitre
mmol	Milimole
ρmol	Picomole
g	Relative centrifugal force
i.p.	Intraperitoneal
rads	Radiations
^{137}Cs	Radioactive isotope of Caesium
^{3}H	Tritium
Ci	Curie
Bq	Becquerel
kDa	Kilodalton
PT	Post treatment
GBq	Gega becquerel
mm	Milimetre
Cm	Centimetre
°C	Degree Celsius
w/v	Weight by volume
v/v	Volume by volume
P	Probability
N	Total number of samples (experiments).
T	Test statistic for two sample T-test
PBS	Phosphate buffered saline
PS	Phosphate saline
PSG	Phosphate saline glucose
CBSS	Carter's Balanced Salt Solution
CPM	Counts per minute
BSA	Bovine serum albumin
DMSO	Dimethyl sulfoxide
CNS	Central nervous system
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
mRNA	Messenger RNA
ELISA	Enzyme linked immunosorbent assay
PCR	Polymerase Chain Reaction
STDM	Standard trypanosome detection methods
OD	Optical density

IU	International units
Min.	Minute
Ads	Adenosine
Deoxyads	Deoxyadenosine
Adn	Adenine
Ino (I)	Inosine
BN	Berenil
TREU	Trypanosomiasis Research Edinburgh University
CTVM	Centre for Tropical Veterinary Medicine

CHAPTER ONE

INTRODUCTION

Trypanosomes are flagellated protozoan parasites belonging to the Order Kinetoplastida, Family Trypanosomatidae and Genus *Trypanosoma*. The parasites are found in the peripheral blood of domestic and wild animals throughout the World. Some species also infect man. Most members of the family Trypanosomatidae are not pathogenic for their host. However a minority of trypanosome species are responsible for causing diseases in animals and man in tropical and sub-tropical areas of the world.

The genus *Trypanosoma* is subdivided into two main sections (the Stercoraria and Salivaria) on the basis of their site of development in the insect vector which transmits them. The stercorarian trypanosomes undergo cyclical development in the hind gut of biting insects following ingestion of an infected blood meal. Infection is transmitted to new hosts by infective metacyclic trypanosomes, via faecal contamination. This section contains one important species, *Trypanosoma cruzi*. *T. cruzi* causes Chagas disease in man and is transmitted by reduviid triatomine bugs. Chagas disease has been rated as the most serious parasitic disease in South and Central America. An estimated 16-18 million people are affected with around 100 million at risk of infection (Guhl and Schofield, 1996; Docampo and Schmunis, 1997). Young people are particularly likely to die in the acute febrile stage. Adults tend to survive to the chronic phase in which the parasite causes damage to the heart muscles and autonomous nervous tissue. Congestive heart failure and dilatation of the oesophagus and colon are common features. There is yet no cure for Chagas' disease. However, presently, the disease is on the decline as a result of vector control (Guhl and Schofield, 1996; Schofield and Dujardin, 1997).

Trypanosomes belonging to the section Salivaria develop in the anterior station as well as the gut of *Glossina* species (tsetse fly) and are transmitted from host to host by infective bite. Several species of trypanosomes that cause important diseases in

animals and man belong to this section. *T. brucei, T. congolense* and *T. vivax* primarily infect cattle, sheep, goats and other domestic animals. They cause a disease condition commonly called nagana in affected animals. These three species constitute a major constraint to the development of the cattle industry in the sub-Saharan Africa. Other minor species include *T. simiae* and *T. suis* which cause infections in pigs. Two members of this section also cause diseases in humans: *T. gambiense* is distributed in West and Central Africa and causes the Western African sleeping sickness. *T. rhodesiense* in East and Southern Africa is the cause of Eastern African sleeping sickness.

Some salivarian trypanosomes are capable of mechanical (non-developmental) transmission, on the mouth parts of other biting Diptera. Important in this category is *T. evansi* which causes a disease known as surra in affected animals, and *T. equiperdum* the causative agent of dourine, a venereal infection in equines; and *T. vivax* in South America.

T. evansi is a monomorphic (i.e. consisting of only one developmental stage) trypanosome species morphologically similar to the long slender trypomastigote form of the pleomorphic *T. brucei* species. Unlike other members of this genus *T. evansi* is mechanically transmitted by Tabanidae and to a lesser extent other blood-sucking insects. In view of its mechanical transmission *T. evansi* has the widest geographical distribution among all the pathogenic trypanosome species. Its distribution is world-wide affecting domesticated livestock in many countries of Africa, Central and Southern Asia as well as Central and South America.

Trypanosomosis caused by *T. evansi* is a problem of great economic importance in affected areas. Important working animals such as camels, horses, buffaloes and cattle are affected. Outbreaks of disease occur sporadically resulting in high levels of morbidity and mortality in endemic areas. The productivity of farm animals such as Friesian Holstein cattle, especially the bull calves, can be adversely affected by *T. evansi* (Payne *et al.*, 1994a).

The use of antitrypanosomal drugs has been the mainstay for controlling animal trypanosomosis. Other control measures aimed at vector eradication such as use of

insecticides or biological methods have been successful only to a limited extent. Furthermore, antigenic variation by trypanosomes has constituted a major barrier to the development of vaccines for the control of trypanosomosis (Overath et al, 1994).

Antigenic variation is the process by which a trypanosome changes its surface glycoprotein coat thereby presenting the host with varying antigen types (Borst and Rudenko, 1994; Barry, 1997). At infection the trypanosome presents a particular antigen to the host against which antibodies are produced. However, by the time the antibodies are produced a smaller number of trypanosomes have replaced their surface coat with one comprising different VSGs (variant surface glycoprotein), and thus are not recognised by these antibodies. A majority of the parasites are destroyed resulting in rapid fall in parasitaemia. However, the few that escaped the antibodies proliferate, presenting a different antigen type to the host. The host again produces antibodies specific to this new type of antigen. This process continues, but the parasite is at least one step ahead of the host each time. Because of the high numbers of variable antigen types in each trypanosome species (Barry and Turner, 1991) it has been impossible to develop a suitable vaccine that could protect animals against any of the forms of trypanosomosis.

Despite the fact that chemotherapy is the major means of controlling trypanosomosis few drugs are available for this purpose. Treatment and prophylaxis of trypanosomosis in cattle, sheep and goats is currently dependent upon the use of three drugs, namely, homidium, a phenanthridine compound, isometamidium, a phenanthridine-aromatic amidine, and diminazene, an aromatic diamidine (Williamson, 1970). Three other compounds are generally used for therapeutic or prophylactic purposes in camels, equidae and buffaloes. These are suramin, a sulphonated naphthylamine; quinapyramine, a quinoline pyrimidine (Williamson, 1970) and melarsomine (mel Cy), a melaminyl thioarsonite (Raynaud *et al.*, 1989a). Some of these compounds have been used for prolonged period of time. Suramin for example, have been in use for over 70 years and, presently commercial production of this drug has been stopped.

There is urgent need for new compounds for the treatment of trypanosomosis. However, development of new antitrypanosomal drugs has been static over the last few decades. This lack of interest has been largely because animal trypanosomosis occurs in developing countries most of which have poor, unsustainable market economies. The resultant small and uncertain market for trypanocidal drugs discourages the significant involvement of the pharmaceutical industry (Gutteridge, 1985). The present emphasis is on effective utilisation of the few existing drugs in order to prolong their useful life. Towards achieving this goal a clear understanding of aspects of drug disposition, mechanisms of action, toxicity and resistance is essential.

Drug resistance was first recognised by Paul Ehrlich with the arsenicals he used to treat trypanosome infections. Drug resistant organisms have been described as stable genotypic variants, selected from a normally sensitive population by drug exposure, that have the ability to survive and multiply in the presence of normally effective drug concentrations (Geary *et al.*, 1986). Today, reports abound of the occurrence of strains of trypanosomes resistant to different trypanocides in different parts of the world. For example, ethidium-resistant *T. congolense* was reported in Nigeria (Ilemobade *et al.*, 1975) and Ethiopia (Scott and Pegram, 1974); isometamidium resistant *T. congolense* is common in Central and West Africa (Pinder and Authie, 1984; Chitambo and Arakawa, 1992); diminazene aceturate resistant *T. congolense* in Zambia (Chitambo and Arakawa, 1992), Tanzania (Mbwambo *et al.*, 1988), and Zimbabwe (Joshua *et al.*, 1995); diminazene aceturate resistant *T. evansi* in China (Zhang *et al.*, 1990) and *T. vivax* in French Guyana (Desquesnes *et al.*, 1995); and suramin-resistant *T. evansi* in Indonesia (Payne *et al.*, 1994a) and Sudan (Abebe *et al.*, 1983). Drug-resistant strains of trypanosomes that affect humans have also been reported. Pentamidine-resistant *T. gambiense* has been reported in Zaire, and *T. rhodesiense* resistant to melarsoprol has been reported in Uganda (Bacchi, 1993). Also *T. rhodesiense* naturally resistant to difluoromethylornithine (DFMO) has been reported from East Africa (Iten *et al.*, 1995).

The problem of drug resistance in animal trypanosomosis has been exacerbated by the frequent misuse of veterinary trypanocides such as irregular administration of

drugs, including prophylactic drugs. This gives rise to the existence of low subcurative concentrations of trypanocides which are suitable for selection of drug resistant mutants. Furthermore, it has been found that strains of trypanosomes that are made resistant to one trypanocidal drug are often cross-resistant to one or more other trypanocides.

A detailed understanding of the mechanisms by which parasites develop resistance is an important starting point towards optimisation of the few available trypanocides. The development of drug resistance is thought to be multifactorial. As such information on the pharmacology of the drug, its mode of action and its biochemical pathways within the organism is required. Some of these vital information are presently lacking for most of the few available veterinary trypanocides as well as the different parasites they affect. The control of *T. evansi* is particularly at risk because commercial production of suramin has ceased. It is anticipated that this situation would lead to overdependence on the very few alternative drugs such as quinapyramine and mel Cy.

Mel Cy is an injectable, trivalent arsenical compound that has been developed in the last decade for treatment of infections with trypanosomes belonging to the *brucei* group (Raynaud *et al.*, 1989a). This drug has been shown to be very effective against *T. evansi* infections in camels, buffaloes, goats and pigs, and *in vitro*. It is reported to be effective against stocks of *T. evansi* resistant to quinapyramine and suramin, and *T. brucei* stocks resistant to diminazene aceturate (Zweygarth and Kaminsky, 1990). Mel Cy has been found useful as part of combination chemotherapies in mouse models of secondary human trypanosomosis (Jennings, 1993). So far there has been no reports of occurrence of field strains of trypanosomes resistant to mel Cy.

This study was essentially an investigation on the mechanisms of acquisition of resistance to arsenical drugs by *T. evansi*, using mel Cy. The *T. evansi* used was a field isolate from Indonesia. This was cloned and adapted to laboratory growth in mice at the Centre for Tropical Veterinary Medicine (CTVM). The overall aim was to elucidate the various adaptations which make it possible for drug-resistant trypanosomes to evade the actions of drugs to which drug-sensitive organisms

succumb. This may highlight possible ways by which such pathways can be by-passed, thereby circumventing resistance. Alternative pathways found may also be explored in the chemotherapy of drug-resistant trypanosomes.

The experimental design was to compare the development of resistance using *in vitro* systems to that in animal models. The ultimate goal was to determine if *in vitro* systems would be useful substitutes for animals in the study of drug resistance.

The specific areas of investigations included the following:

1. Induction of resistance to mel Cy in *T. evansi* using different laboratory methods in order to determine the range of sensitivities expressed by populations derived from the parent clone.

2. Characterisation of the resistance induced by mel Cy in terms of the behaviour of resistant parasites in the host, stability of resistance, and cross-resistance to other trypanocides.

3. Experiments to ascertain whether mel Cy and mel B would cause lysis of *T. evansi* similar to that observed with other arsenical drugs in related trypanosome species.

4. Determine to what extent this *in vitro* lysis can be used to differentiate between drug sensitive and drug resistant trypanosomes.

5. Identification of compounds which inhibit lysis of *T. evansi* by mel Cy and mel B with a view to determining the mode of uptake of these arsenicals drugs.

6. Determination of the mechanisms of resistance in mel Cy through a detailed study of the purine transport pathways of the drug-resistant clones in comparison with the drug-sensitive isolate.

7. Investigation of the influence of the host environment on the mechanisms of resistance development.

CHAPTER TWO

LITERATURE REVIEW

2.1. TRYPANOSOMA EVANSI

2.1.1. Historical

It is believed that the first observations on trypanosomes were made by Valentin in 1841 who described findings in the blood of trout (*Salmo fario*) of motile elongated organisms which he likened to *Amoeba*. However, the true nature of these haemoflagellates was recognised by Gruby in 1843 who created the generic name *Trypanosoma* (Hoare, 1972). *Trypanosoma evansi* was the first trypanosome to be described and identified as the causative agent of mammalian trypanosomosis. Earliest reports on the trypanosome were published by Evans (1880, 1881- 1882) who associated the parasite with an endemic disease in equines and camels, known as "surra" in the Dera Ismail Khan in Punjab in India. Steel (1885) made similar observations during an outbreak of a disease in transport mules in Burma and he named the parasite *Spirochete evansi*. Its true nature was recognised by Crookshank (1886), and Steel's name was amended to *Trypanosoma evansi* by Balbiani (1888) (Hoare, 1970). Trypanosomosis due to *T. evansi* (= *T. ninaekolyakimovi*, Hoare, 1972) was also later described by Yakinoff, 1921-1923, in Russian camels in Turkestan. In his report Yakinoff stated that trypanosomosis in Russian camels was first detected by Feinschmidt in 1912 in Astrakhan and Saratov (reviewed in Mahmoud and Gray 1980). Rogers (1901) proved experimentally that surra in India was transmitted mechanically by tabanid flies (Hoare 1972).

Following these early reports more discoveries were made of diseases caused by trypanosomes indistinguishable from *T. evansi* in different mammalian hosts in different parts of the world. Thirty three different names have been associated with these trypanosomes but *T. evansi* is accepted as the valid name for the parasite of

surra. The name, *T. evansi* now applies to the trypanosomes causing "murrina" (*T. hippicum*), "Mal de Caderas" (*T. equinum*) , "Derrengadera" (*T. venezuelensis*) in Central and South America and "Su-auru" (*T. ninaekolyakimovi*) in Russia (Hoare, 1972; Mahmoud and Gray, 1980).

2.1.2. Geographical distribution

T. evansi is widely distributed in countries with hot and warm-temperate climates. Details of its geographical distribution is given by Hoare (1970, 1972). In North Africa *T. evansi* occurs along the Atlantic and Mediterranean regions. It is prevalent in Morocco, Algeria, Tunisia, Libya and Egypt. In the West it extends across the Sahara into Senegal, Mali, Chad and other parts of West Africa, north of the tsetse belt varying between latitudes 13^{0}N in Nigeria to 15-16^{0} N elsewhere. In the East the disease spreads southwards to about 13^{0} N in Sudan and almost up to the equator in northern Kenya and Somalia.

In the Eurasian continent distribution of *T. evansi* is uneven and patchy. In the Near and Middle East surra has been reported from Israel, Lebanon and Syria, the northern half of the Arabian Peninsula, Asia Minor, Iraq and Iran. In Europe Turkey and Bulgaria are affected, but the trans Volga region of the Soviet Union is mostly affected. The prevalence extends to the Soviet Middle East, Iran, India, Burma, Malaysia, Indo-China and parts of southern China. The parasite also occurs in islands of the Indian Ocean (Mauritius, Reunion), Indonesia and Philippines.

In the new world *T. evansi* (Murrina, syn: *T. hippicum*) is found in Mexico, all of Central America, Venezuela and Colombia while Mal de Caderas (syn: *T. equinum*) is present in a greater part of South America, especially Brazil with Derrengadera (syn: *T. venezuelensis*) occupying an intermediate position (Hoare, 1972; Lun and Desser, 1995).

2.1.3. Host range

T. evansi occurs in a number of domestic and wild animals. Its principal hosts are camels, equines and dogs, but it is common in bovines and it has been found in the

Indian elephant (Hoare, 1972). Although *T. evansi* is pathogenic to all domestic animals its effect upon the host depends upon both the virulence of the strain of parasite and the susceptibility of the host (Hoare, 1956, 1970). Camels are highly susceptible to infection and the disease in this species usually runs an acute or chronic course, terminating fatally in untreated cases. Horses are similarly affected in North Africa whereas in Sudan and Somalia they are refractory to infection. The disease in cattle and pigs is mild and often asymptomatic while in dogs the course of infection is acute (Hoare, 1970).

T. evansi epidemics often involve different animal hosts in different parts of the world. In Indochina horses are mainly affected, followed by bovines and buffaloes, whereas in the Soviet Middle Asia the main hosts are camels, and to a lesser extent horses. In Africa camels are mostly affected while in Central and south America horses are the main hosts followed by cattle (Mahmoud and Gray, 1980). Natural infections have also been reported in mules and donkeys (Mahmoud and Gray, 1980). Evidence from experimental infections confirm that donkeys, cattle sheep and goats undergo protracted course of disease that results in a "carrier state" and may act as reservoir hosts (Ilemobade, 1971; Mahmoud and Gray, 1980).

Healthy Capybaras (*Hydrochoerus hydrochaeris*) have been shown to harbour *T. evansi* and can constitute a reservoir of infection for horses and dogs in Colombia (Morales *et al.*, 1976). Similarly, Ocelot (*Felis pardalis*) in the lower Amazon Region and Orangutan (*Pongo pygmaeus*) in Sumatra. Experimental infections have been established in monkeys (Family Cercopithecidae) with sub-clinical symptoms. *T. evansi* is pathogenic to all laboratory mammals which rapidly succumb to the infection (Hoare, 1970). The parasite has been shown to be susceptible to human plasma, hence is of no zoonotic importance (Hawking, 1978).

2.1.4. Morphology

Detailed investigation into the morphological characteristics of various strains of *T. evansi* from different parts of the world was carried out by Hoare (1956). A detailed description of the morphology of the parasite is given in Hoare (1972).

T. evansi is generally regarded as monomorphic. It is represented almost exclusively by thin trypomastigotes comprising the slender and intermediate forms identical to those of *T. brucei*. The slender forms have a long free flagellum, and a drawn out narrow posterior extremity which may be rounded or truncated, with the kinetoplast situated at some distance from the tip. The intermediate forms have a shorter free flagellum and a short, frequently pointed posterior extremity, with the kinetoplast lying near this end. The kinetoplast of *T. evansi* is small, usually rod-shaped and typically sub-terminal or marginal in position (Hoare, 1972) (see Plate 2.1).
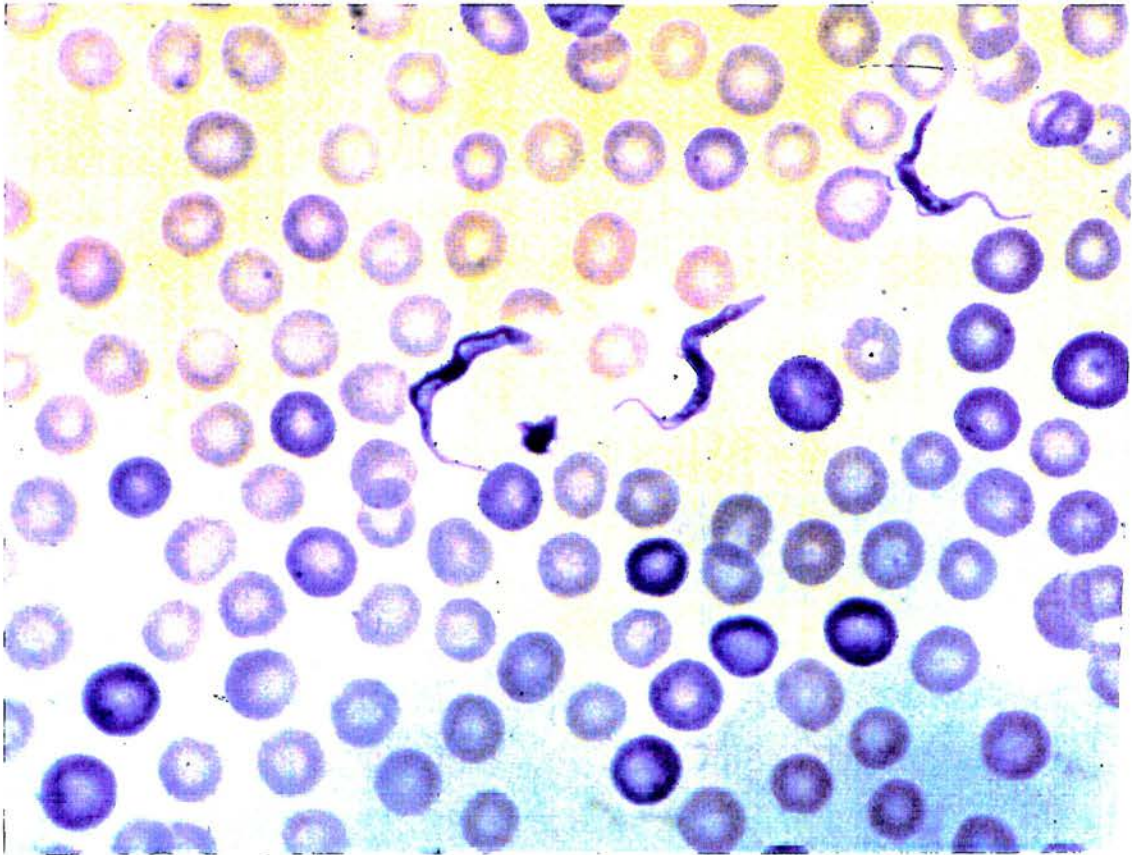
Plate 2.1: Micrograph showing *T. evansi* in peripheral blood. Giemsa stain, x400 magnification.

2.1.5. Transmission

Unlike most other species of *Trypanosoma*, *T. evansi* does not undergo any cycle of development in an intermediate host but is transmitted mechanically from one host to another by blood-sucking Diptera belonging to the family Tabanidae. In this case trypanosomes are merely transferred from an infected to a susceptible host by the interrupted feeding of the biting fly. The trypanosomes in or on the contaminated proboscis of the fly do not multiply and die quickly so cross transmission is only possible for a few hours (Urquhart *et al.*, 1996). It has been shown that transmissibility is highest when the interval between the feeding on the donor and recipient did not exceed 15 minutes, and no infection was produced when the interval exceeded 8 hours (Hoare, 1972).

Various species of *Tabanus* (Horse fly) and *Stomoxys* (Stable fly) have been shown to transmit *T. evansi*, *Tabanus* species being the most efficient transmitters (Luckins, 1988). *Tabanus* species are important in transmission under field conditions, while transmission in stables is effected mainly by *Stomoxys* species (Hoare, 1970, 1972). It has been demonstrated experimentally that *T. evansi* is incapable of undergoing cyclical development either in tabanids or other flies, including *Glossina spp* (Hoare, 1972).

In Central and South America, in addition to tabanid flies, *T. evansi* has also been proven (both experimentally and under natural conditions) to be transmitted by the bites of sanguinivorous vampire bat (*Desmodus rotundus*). These bats which are widespread in Latin America (extending from northern Mexico to Southern Argentina) become infected when feeding on infected horses or cattle. The disease which usually lasts for about one month may terminate fatally or the bat may survive and recover spontaneously. Trypanosomes gain access into the bloodstream of the bat through penetration of the mucous membranes of the buccal cavity. The parasites multiply in the blood however, without undergoing any morphological transformation. They then migrate back into the buccal cavity where they are inoculated into a new host while the animal takes its blood meal. In effect the bat plays a dual role of being both the vector and the host for *T. evansi* and thus is

capable of transmitting the parasites for a relatively long period of time. This particularly makes them very important in the spread of *T. evansi* in those regions. Carnivores, especially dogs and wild Carnidae become infected with *T. evansi* by ingestion of infected carcass.

2.1.6. Classification

A detailed classification of *T. evansi* is given in Table 2.1.

T. evansi can be distinguished from other members of the subgenus *Trypanozoon* using essentially three criteria: absence of tsetse flies in the area where the parasite was isolated; absence or rarity of pleomorphism in the bloodstream forms; and, the inability of the trypanosome to develop in *Glossina* (Hoare, 1970).

Hoare (1970) suggested that *T. evansi* descended from *T. brucei* when the latter was carried outside the tsetse belt by camels. Subsequent repeated transmission over many years from host to host by biting flies such as tabanids may have transformed the parasite into the *T. evansi* known today, and the replacement of *Glossina* by mechanical inoculations such as Tabanidae. This enabled the disease surra to spread to outside the tsetse belts to the north of Africa and beyond the continent to Europe, Asia and the Americas.

2.1.7. Diseases caused by *Trypanosoma evansi*

The clinical course of *T. evansi* infections depend on the susceptibility of the host and the strain of the parasite. Generally the symptoms closely resemble those caused by *T. brucei.*

In horses the acute disease may be as short as 2-3 weeks, chronic phase lasting up to four months. Clinical signs include pyrexia, progressive anaemia, ascites and oedema of the ventral abdomen and genitalia. There is a characteristic transient appearance of small urticarial plaques which coincides with peak parasitaemia, and petechiation of mucous membranes. Camels are readily infected by *T. evansi*. The first clinical sign is pyrexia followed by anaemia. The course of the disease varies with the strain of the parasite and the physical condition of the animal which is related to the

Table 2.1: Classification of *Trypanosoma* (According to Hoare, 1970).

Kingdom: Protista
Phylum: Protozoa
Subphylum: Sarcomastigophora
Superclass: Mastigophora
Class: Zoomastigophora
Order: Kinetoplastida
Suborder: Trypanosomatina
Family: Trypanosomatidae
Genus: *Trypanosoma*

Section:	Stercoraria			Salivaria			
Subgenus:	*Megatrypanum*	*Herpetosoma*	*Schizotrypanum*	*Duttonella*	*Nannomonas*	*Trypanozoon*	*Pycnomonas*
Species:	T. theleri	T. lewesi	T. cruzi	T. vivax	T. congolense	T. brucei	T. suis
				T. uniforme	T. simiae	T. rhodesiense	
						T. gambiense	
						T. evansi	
						T. equiperdum	

nutritional status and the amount of work which it is required to do. The disease in horses in South America, known as *mal de caderas* (meaning disease of the hip) and *murrina*, is characterised by signs of a gradual development of central nervous system involvement, and progressive paralysis of the hind quarters is common. As the disease advances there is increasing weakness, oedema of dependent parts and marked loss of condition.

Cattle and Asiatic buffalo are readily infected with *T. evansi* but they seldom show clinical signs. The disease may, however, flare up in acute or peracute form. Cattle are important reservoir hosts in Asia from where more susceptible species such as horses and camels may become infected. African cattle on the other hand appear to be rarely infected with *T. evansi* even in situations where they have been in close contact with infected camels (Hoare, 1972; Leach and Roberts, 1981).

Dogs are occasionally infected and the course of infection is usually acute and generally fatal if not treated. In addition to the other typical symptoms of trypanosomosis the parasite may invade the eyes, causing conjunctivitis and keratitis, and occasionally affecting the lens and the iris.

2.2. IN VITRO CULTIVATION OF TRYPANOSOMES

Cultivation of trypanosomes in culture medium has been done since the beginning of the twentieth century (Novy and MacNeal, 1903). Initially, it was only possible to cultivate the insect forms of the cyclically-transmitted trypanosomes by selecting culture media and temperature (26^{0}C) that mimic their growth requirements in the insect vector (Brun and Schonenberger, 1979). For this reason it was believed that *in vitro* cultivation of the mechanically transmitted species such as *T. evansi* was not possible as there are no insect forms. This was also considered to serve as a tool for their characterisation (Hoare, 1972).

Effective long-term culture systems for cultivation of bloodstream forms using feeder-layer cells were later developed for *T. brucei* (Hirumi *et al.*, 1977), *T. vivax* (Brun and Moloo, 1982), T. *congolense* (Gray *et al.*, 1985; Ross *et al.* 1985) and *T.*

gambiense (Yabu *et al.*, 1986; Yabu and Takayanagi, 1986,1987; Yabu *et al.*, 1989). These studies showed that mammalian feeder cells were essential for the *in vitro* growth of African trypanosome bloodstream forms at 37^0C. The trypanosome growth-supporting activity of both serum and feeder layer has been found to vary considerably, even for different strains of the same trypanosome species. Thus optimum culture conditions have to be established for each strain (Borowy *et al.*, 1988).

Despite the progress made in the cultivation of cyclically transmitted stocks of the subgenus *Trypanozoon*, both with the procyclic (Brun and Schonenberger, 1979) and bloodstream forms (Hirumi and Hirumi, 1990; Hesse *et al.*, 1995), *T. evansi* was for a long time cultivated successfully only in embryonated eggs or chick embryos and not in a variety of culture media. Later on a biphasic culture system consisting of rabbit fibroblasts feeder layer in a modified RPMI 1640 medium was developed for *T. evansi* (Zweygarth *et al.*, 1983).

A significant improvement in the cultivation of *Trypanozoon* species resulted from the findings of Baltz *et al* (1985) that a major function of the feeder layer for such parasites was its reducing capacity and that it can be replaced by reducing agents such as 2-mercaptoethanol. About the same time Duszenco *et al* (1985) found that feeder cells promote the growth of African trypanosome bloodstream forms by continuously supplying cysteine and he axenically cultured *T. brucei* bloodstream forms successfully by adding cysteine to the culture medium.

The major problem with this innovation was the rapid rate of cysteine oxidation during incubation at 37^0C. In mammalian cells the copper ion was identified as the major catalyst for oxidation of cysteine in serum-supplemented culture media and a non toxic, copper-specific chelating agent, bathocuproine sulfate (BCS) was found to inhibit the autoxidation of cysteine (Duszenco *et al.*, 1985). Yabu *et al.*, (1989) demonstrated that axenic culture system supplemented with cysteine and BCS was indeed effective for supporting long-term culture of *T. gambiense*. Axenic suspension culture systems are now used for propagation of other *Trypanozoon*

species and this system has been effective in long-term maintenance of *T. evansi* cultures.

2.3. DIAGNOSIS OF ANIMAL TRYPANOSOMOSIS

Several factors are important in considering the presence of trypanosomosis. these may be based on epidemiological, serological or clinical evidence. Knowledge of the geographical distribution of the parasite is essential in identifying areas of endemicity but diagnosis of trypanosomosis ultimately depends on the demonstration and identification of the parasite in the host (Leach and Roberts, 1981). Thus sensitive and reliable techniques for the diagnosis of infection are essential to ensure the detection of infected animals and the effective application of chemotherapeutic control measures. Furthermore in epidemiological studies it is often necessary to determine the prevalence and incidence of parasites. Thus parasitological techniques are often combined with immunological techniques to distinguish animals with active infections from those which have experienced the disease but may have been cured. This presentation looks briefly at the clinical, parasitological and immunological aspects of diagnosis and some methods of characterisation relevant to *T. evansi.*

2.3.1. Clinical diagnosis

Epidemiological evidence of recent contact with possible vectors is an important indication of the presence of trypanosomosis. This is more applicable to the tsetse-transmitted trypanosome species rather than *T. evansi.* This is because the tsetse belts in most endemic areas are well defined. The vectors of *T. evansi*, however are not confined to well-defined zones and history of contact may not be properly established. Chronic trypanosomosis and the wasting process associated with it may, however, take several months to manifest itself and animals may have been removed from the source of infection some months before the development of clinical signs.

Acute trypanosomosis may involve rapid loss of condition of affected animal(s), sudden death in a group, acute febrile reactions and, trypanosomes are easily detectable in the blood. However mild acute infections may pass undetected until

they go into chronic stage and only loss of condition may prompt investigation, or infections may be found in routine field surveys (Leach and Roberts, 1981).

2.3.2. Parasitological techniques

Parasitological techniques comprise methods for detecting the parasites in the blood of infected host. These methods which are used either in the field or laboratory are divided into two: The Standard Trypanosome Detection Methods (STDM) (Molyneux, 1975) include the wet, thin and thick blood smears, which are simple but relatively less sensitive methods, requiring less sophisticated laboratory equipment and utilising small volumes of blood; haemoconcentration and rodent inoculation methods. The other techniques which are more suitable for laboratory or experimental survey work include culture and column separation methods.

Wet blood film examination (Baker, 1970) is particularly useful for routine examination of experimental animals (Leach and Roberts, 1981). In this method a drop of blood obtained from an infected animal is placed on a clean microscope slide and a coverslip placed on the drop of blood to allow an even spread on the slide. This is examined at x40 objective of a light microscope. Usually 60-100 microscope fields are examined (Killick-Kendrick, 1968).

Stained thick blood smears are more useful for field work (Killick-Kendrick and Godfrey, 1963). The method of preparation is described by Maclennan (1957). The main disadvantage of thick blood smear is distortions of trypanosomes during the staining process which can cause difficulty in identification.

Thin blood smears are useful in the identification of species of trypanosomes (Killick-Kendrick, 1968), although they have been considered to be far less sensitive than the thick smears (Fiennes, 1952).

Simple concentration methods such as the Haematocrit Centrifugation Technique (HCT) (Woo, 1970) have increased sensitivity over examination of blood smears in the diagnosis of trypanosomosis. In the HCT suspected blood is centrifuged in a micro-haematocrit and trypanosomes are examined for at the plasma- buffy coat interface either by direct inspection through the walls of the capillary tube, using a

phase-contrast microscope, or by breaking the tube and making smears of the interface material. The efficiency of this method in detecting *T. brucei*, *T. evansi* and *T. rhodesiense* is estimated to be 85%. The method is capable of detecting parasitaemias as low as 1×10^2/ml of blood (Woo and Rogers, 1974).

The rodent inoculation method consists of sub-inoculation of whole blood into rodents and periodic examination of blood from inoculated animals for 1-2 months following injection. This technique is most sensitive for detection of trypanosomes of the subgenus *Trypanozoon* which are normally present in blood in low numbers (Woo, 1970) and is found to be most effective at detecting sub-patent infections of *T. evansi* (Payne *et al.*, 1990). However this method suffers a number of disadvantages. It is not convenient for use on the field due to the difficulty of maintaining large numbers of rodents in the field. Several species of trypanosomes are either refractory to rodents or are of low infectivity, or cause only transient infections (Godfrey, 1961; Roberts and Gray, 1973; Leeflang *et al.*, 1976). Laboratory animals are not often available in endemic areas, hence this method is not used regularly on the field. It is however very convenient for laboratory use.

Culture of suspected blood for the diagnosis of pathogenic trypanosomosis in animals is not a satisfactory method because of the high risk of contamination by non-pathogenic trypanosomes of the sub-genus *Megatrypanum*, such as *T. theileri*; and bacteria (Hoare, 1972; Lanham, 1977).

The DEAE-cellulose column separation technique (Lanham and Godfrey, 1970) is very useful in the laboratory for isolating large numbers of trypanosomes from blood in pure suspensions for biochemical work. In this method red blood cells are eliminated from the blood sample by passing it through an anion exchange column. The erythrocytes which are negatively charged than the trypanosomes attach to the column but the positively charged trypanosomes pass through and are subsequently concentrated when the eluate is centrifuged. A miniaturised column was developed for processing small quantities of blood (50-100 µl) (Lumsden *et al.*, 1979). However the high cost and complexity of this technique does not permit its routine use for diagnosis of animal trypanosomosis (Leach and Roberts, 1981).

Although parasitological techniques have been used in studies on infection with *T. evansi* the major disadvantage with these methods have been that trypanosomes are regularly absent from circulation due to oscillations in parasite populations. In a survey of camel blood for *T. evansi* Godfrey and Killick-Kendrick (1962) showed that the rat inoculation test demonstrated parasites in 27.6% of the animals while microscopical examination of thick blood films detected parasites in only 12.4%. In a similar survey in India *T. evansi* was detected in 10.09% and 11.46%, respectively, in buffaloes and cattle examined using HCT whereas the examination of blood films revealed parasites in only 1.44% and 0.48% of buffaloes and cattle sampled (Payne, 1989).

After demonstration of parasites in the blood characterisation of the particular trypanosome species is achieved on the basis of morphology, biochemical or molecular characteristics.

2.3.3. *Characterisation using isoenzyme analysis*

Isoenzymes are multiple molecular forms of the same enzyme which usually bear similar properties but differ in their electrophoretic mobilities due to differences in electric charge caused by variations in their primary, secondary or tertiary molecular structures. The primary variations have been classified as structural while the secondary and tertiary variations are classified as post-translational. Structural changes are known to occur at the level of transcription when the genetic codes of various polypeptides that make up an enzyme are determined. Post-translational changes are caused by modifications which occur after the polypeptide chain has been synthesised and lead to the final form of the enzyme. Structural or post-translational changes may not change the function of the resulting enzymes although they may result in changes in the overall net charge leading to differences in electrophoretic mobilities (Harris and Hopkinson, 1976).

Isoenzyme systems and their electrophoretic mobility differences have been used to characterise different species of parasites including *Trypanosoma* (Murray, 1982; Godfrey *et al.*, 1987). Isoenzyme analysis has been particularly useful in differentiating parasites of the subgenus *Trypanozoon* which are morphologically

indistinguishable (Gibson *et al.*, 1980; Gibson and Wellde, 1985; Godfrey *et al.*, 1990). Several enzymes have been identified in this subgenus which are useful in distinguishing the various species. Of these 5 enzymes, namely, aspertate aminotransferase (ASAT), phosphoglucomutase (PGM) malic enzyme (ME), peptidase 1 (PEP1) and peptidase2 (PEP2) have been found to vary in different isolates of *T. evansi* from camels. These have been used to differentiate *T. evansi* from other members of the *Trypanozoon* subgenus (Gibson *et al.*, 1980). Five patterns of malic enzymes have so far been identified in *T. evansi*. (Gibson 1983; Boid, 1988).

2.3.4. Characterisation using molecular methods

Current available techniques are able to characterise trypanosomes based on differences in their genetic material which comprise the deoxyribonucleic acid (DNA). Specific DNA fragments (probes), have been shown to be important in characterising these parasites. These fragments are labelled either with radioactive material or enzymes and the degree of similarity in test DNA samples is determined by hybridisation reactions.

Molecular methods have been used to distinguish *T. evansi* from other members of the subgenus *Trypanozoon*. These have been based mainly on the differences in kinetoplast DNA between *T. evansi* and other members of the subgenus (Borst and Hoeijmakers, 1979; Borst *et al.*, 1987). The kinetoplast of the cyclically-transmitted *Trypanozoon* parasites contains two DNA components consisting of 50-100 homogeneous maxi circles and 5,000-100,000 heterogeneous mini circles. *T. evansi* and *T. equiperdum* differ from these other species in that although they have the same number of mini circles these are homogeneous. In addition *T. evansi* completely lacks maxi circles while *T. equiperdum* has maxi circles which are non functional (Borst and Hoeijmakers, 1979). These difference distinguish *T. evansi* from the other morphologically identical species.

Species-specific DNA fragments consisting of kinetoplast DNA (kDNA) sequences (Masiga and Gibson, 1990) have been identified in *T. evansi* which have been found

to be absent in all other trypanosome species. In this test a trypanosome sample is hybridised with a probe on a blot to determine if the species is *T. evansi.*

Another DNA-based method of characterisation is the polymerase chain reaction (PCR). This is an *in vitro* method that is used to amplify a segment of DNA that lies in between two regions of known sequences (primers) (Mullis and Faloona, 1987; Saiki *et al.,* 1988). These known sequences found on the DNA template are recognised by the polymerase enzymes responsible for replication of the DNA as regions from where replication is initiated. Species-specific primers have been identified in *T. congolense* and *T. brucei* (Moser *et al.,* 1989) and *T. evansi* (Masiga *et al.,* 1992). In this method the two strands of a DNA test sample are separated (denatured) by heating at appropriate temperature. This is incubated with a species-specific primer in the presence of DNA polymerase. If the sample is from the homologous species there is replication and a new strand of DNA is synthesised. By raising the temperature again the DNA template together with the newly synthesised DNA are both denatured and lowering of the temperature again results into the synthesis of new DNA using both the original and the new DNA as templates. As this cycle is repeated more DNA is amplified. The reaction mixture is then run on electrophoresis agarose gel where the intensity of the DNA band is compared to the original test sample to determine whether DNA has been amplified.

2.3.5. Immunodiagnostic techniques

Immunodiagnostic approaches to the diagnosis of trypanosomosis are dependent on the fact that parasites elicit an immune response in their host and the detection of the antibodies produced is evidence that an infection has taken place. The range of serological tests for diagnosis of various trypanosomes has been reviewed by a number of authors (Killick-Kendrick, 1968; Molyneux, 1975; Nantulya, 1990; Luckins, 1992). The major limitation of serological tests is that antibodies persists in the host after parasites have been eliminated by chemotherapy (Luckins *et al.,* 1979). Thus the true infection status of a host can not be determined by the mere presence of antibodies which could only indicate previous infection. Another obstacle to the

widespread use of serological techniques in animal trypanosomosis has been the vast diversity of antigens present in the trypanosome species (Gray and Luckins, 1976).

Trypanosome antigens can be divided into 2 groups: those which comprise the surface coat of the organism and the internal antigens underlying the surface coat. The surface coat comprise glycoproteins which are renewed each time the trypanosome undergoes antigenic variation. A specific immune response of the host is mounted against each new trypanosomal variant antigen (Cross, 1975). However, because of their variant specificity such antigens will only detect antibodies produced against homologous variants thus making them of little value in serodiagnostic tests. Wilson and Cunningham (1971) found that the level of serum antibody to heterologous antigen rarely reached positive values in the indirect fluorescent antibody test (IFAT). Serological techniques are however, becoming increasingly useful in the diagnosis of trypanosomosis

2.3.5.1. *Serological tests for Trypanosoma evansi*

A number of chemical tests which depend on increased serum globulin content have been described for *T. evansi* infection. These include the mercuric chloride test, the formol gel test, and the thymol turbidity test (Luckins *et al.*, 1979)

2.3.5.1.1. *The mercuric chloride test*

Mercuric chloride test has been used in the diagnosis of *T. evansi* infections in camels. In this test a drop of the test serum is added to 1 ml of 1:25,000 aqueous mercuric chloride solution. Positive serum forms a fine precipitate within a few moments, but negative samples produce no precipitate after standing for 15 minutes. Degrees of opalescence are recorded from +- to +++. The reaction with camels infected *T. evansi* develops within 2-3 weeks of infection, remains positive throughout but becomes negative after successful treatment (Killick-Kendrick, 1968). A modification of this method was used in camels in Ethiopia and proved more sensitive for detection of *T. evansi* than other tests, apart from rodent inoculation (Pegram and Scott, 1976). Sera from camels infected with *T. vivax* and *T. brucei* also gave positive results.

The mercuric chloride test is thought to be due to a peculiar immune response of camel to trypanosomosis (Killick-Kendrick, 1968). Conflicting reports exists, however, as to the success of this technique in the diagnosis of *T. evansi* infections in camels (Leach, 1961) and the test has not proved useful in other species of animals apart from camels. (Killick-Kendrick, 1968).

2.3.5.1.2. *The formol-gel test*

This test is carried out by adding 2 drops of 40% formaldehyde to 1 ml of serum and allowing the mixture to stand overnight. Positive serum forms an opaque gel. This test although, used for several years for routine diagnosis of *T. evansi* infections was later replaced by the quicker and relatively more reliable mercuric chloride test. The formol-gel test was also applicable only to camels (Killick-Kendrick, 1968).

2.3.5.1.3. *Indirect haemagglutination test*

This test relies on the agglutination in the presence of antibody, of tanned erythrocytes previously coated with soluble antigens obtained from disintegrated trypanosomes collected from blood of experimentally infected rodents. The test was shown to be highly sensitive in diagnosing experimental *T. evansi* infections (Gill, 1964) and is especially useful in bovine and equine species (Killick-Kendrick, 1968).

2.3.5.1.4. *Complement fixation test*

The complement fixation test has been used for routine diagnosis of *T. evansi, T. cruzi* and *T. equiperdum* infections (Leach and Roberts, 1981). In this tests complement is "fixed" by the antigen-antibody complex. An indicator consisting of sheep red blood cells and anti sheep red cell serum is added. If complement is not fixed, the red cells are haemolysed. But if complement was fixed the red cells precipitate, indicating the presence of antibodies in the test serum.

2.3.5.1.5.   Indirect fluorescent antibody test (IFAT)

The IFAT has been used as a rapid screening test. It has been found useful for diagnostic purposes following drug prophylaxis, and is reported to be a more useful tool for surveys than the STDM (Zwart *et al.*, 1973).

In IFAT antigens are prepared from blood smears, fixed in formaldehyde or acetone and reacted with test sera. If antibody is present binding occurs which is visualised using a fluorescent microscope after staining with a specific antiserum conjugated to a fluorescent dye. The results are read and scored according to the intensity of fluorescence. This test has been used to detect trypanosome antibodies in cattle and camels. The tests, however, may not discriminate between trypanosome species (Luckins *et al.* 1979; Luckins, 1992). The main disadvantages of IFAT are that the test requires expensive and sophisticated equipment, and relies on subjective comparison of results. Like other serological tests the IFAT only indicates that an animal had been infected with a trypanosome but does not determine its current status of infection.

2.3.5.1.6.   Enzyme-linked immunosorbent assay (ELISA)

Another important serological technique employed in the diagnosis of *T. evansi* is the enzyme-linked immunosorbent assay (ELISA). In this assay the test serum is reacted with a trypanosomal antigen which is adsorbed onto polystyrene plates. The antigen-antibody complex is incubated with enzyme-conjugated antiglobulin to the IgG fraction of the particular animal species. The test is visualised by the addition of enzyme substrate and chromogen and the presence of antibody is indicated by the degree of substrate degradation. This is measured quantitatively by the resulting colour change, in a spectrophotometer (Luckins, 1977).

The ELISA has several advantages over IFAT, including increased sensitivity, simpler equipment and the elimination of the subjective bias in interpretation. Luckins *et al.* (1979) compared the ability of five tests to detect both experimental and natural infections of *T. evansi* in camels in the Sudan. They found that the correlation of positive results obtained by assays with IgM levels, the mercuric

chloride test and the formol-gel test with the presence of active infection was unsatisfactory. However there was good correlation between results obtained using IFAT and ELISA and proven infection. ELISA also has the added advantage of being suitable for screening large numbers of samples (Luckins, 1977). However, ELISA also suffers a major disadvantage, as in IFAT, in that the test cannot differentiate between past and present infections.

2.3.5.1.7.   *Antigen-ELISA*

A modified ELISA suitable for detecting circulating antigens in the blood of infected animals was developed by Rae and Luckins (1984). Circulating antigens comprise the products of dead and degraded trypanosomes. These were found to be detectable in rabbits infected with *T. evansi* or *T. congolense* 8-14 days after infection, with antigen levels increasing progressively for 50-60 days after infection. Antigens were not detectable in serum 7 days after trypanosomal drug treatment (Rae and Luckins, 1984). In antigen ELISA a trypanosome-specific antibody is used to coat a polystyrene plate. Test serum is added onto the plates and the antigen in serum captured by the coating antibody. A second antibody which is enzyme-labelled is introduced which binds to the captured antigen. The reaction is visualised by addition of substrate and chromogen as described for antibody ELISA.

The antigen ELISA is particularly useful to differentiate animals giving positive reactions in serological tests as a result of persistence of antibodies after drug treatment, and to identify animals with active infection. The antigen ELISA has been found to correlate well with parasitological tests such as microscopy and mouse sub-inoculation (Nantulya *et al.*, 1989) and is considered the best available serological test for the detection of current infection (Mutugi, 1993).

2.4. *CONTROL*

Control of vector-borne parasitic diseases is generally targeted towards interference with the life cycle of the parasite involved, thereby breaking the transmission cycle and limiting movement of parasite from host to host. Control methods used in trypanosomosis are broadly categorised into three, namely methods directed against

the vectors, the parasite or methods involving the host. The other two categories of control methods have been directed mainly towards control of the tsetse-borne diseases. This review looks at methods directed against the parasite.

2.4.1. Control methods directed against the parasite

Elimination of the source of infection carried by the insect vector is the most practical method of controlling infections from non-cyclical transmissions such as *T. evansi*, which is dependent on the existence of infections and close contact between individual animals within the affected herd. This is achieved by drug treatment of affected animals. Moreover it is very difficult to control or eliminate non-cyclical vectors over large areas because these are widespread, and sometimes exceedingly numerous in the environment within which most livestock live. Chemotherapy, therefore remains the only practicable method of controlling these infections in livestock.

2.5. CHEMOTHERAPY AND CHEMOPROPHYLAXIS

2.5.1. Introduction

The use of trypanocides in the treatment and protection of animals is the most common single method employed in the control of animal trypanosomosis. More recent reviews of drugs active against trypanosomes, including the evolution of trypanocides is given by Apted (1980) Leach and Roberts (1981), Losos (1986). The effectiveness of chemotherapy and chemoprophylaxis of animal trypanosomosis depends on a number of factors which include the species of trypanosomes causing the infection, and the occurrence of resistant strains in the area (Losos, 1986).

Based on chemical structure the trypanocides currently used in livestock can be divided into acid naphthylamine, phenanthridine, quinaldine and diamidine groups (Losos, 1986). The most commonly used drugs against *T. evansi* infections are described below.

2.5.2. Suramin

Suramin is a synthetic trypanocide belonging to the naphthylamine group (Figure 2.1). This drug was developed in Germany during the World War II (1914-1918) and became available in 1920. It was shown by Knowles (1925) to have activity against experimental infections with *T. equiperdum* in horses and naturally occurring *T. evansi* infections in camels, and in other species by Edwards (1926).

Suramin is a strongly ionic drug and binds readily to plasma proteins. It is excreted slowly and traces can be found in the blood up to 3 months following intravenous administration, which accounts for its prophylactic activity (Dewey and Wormall, 1946). Suramin does not enter erythrocytes, nor cross the blood-brain barrier, hence it is ineffective in the treatment of infections involving the central nervous system (CNS). Suramin is administered intravenously as it causes severe local reactions when given by other routes. It has no pronounced activity against *T. vivax* and *T. congolense* infections of cattle and has only a therapeutic effect on *T. simiae* in pigs (Stephen, 1966). With the exception of quinapyramine, suramin has greater efficacy against the *Trypanozoon* subgenus compared to most of the modern trypanocides which are mostly used in cattle. It had been for a long time the drug of choice in the treatment of *Trypanozoon* infections of equidae (*T. brucei, T. evansi and T. equiperdum*) as it is less toxic in equines compared to quinapyramine. The main use of suramin has been the treatment of *T. evansi* infections in camels until the later innovation of quinapyramine 1946-1950 (Williamson, 1970). Single intravenous doses of 5-10g suramin in aqueous solution were curative but conferred only short prophylactic effect of 1-4 months, and repeated use of small doses led to development of drug resistance. Suramin-resistant *T. evansi* is susceptible to quinapyramine treatment. Suramin was not replaced by the newer cattle drugs mainly because the latter has been found to be either far less effective, or, like quinapyramine, cause more severe toxic reactions in horses than in cattle.

Suramin may cause delayed toxicity, including nephritis. The drug has been shown to aggregate in lysosomes (Smeethers and Jacques, 1968).
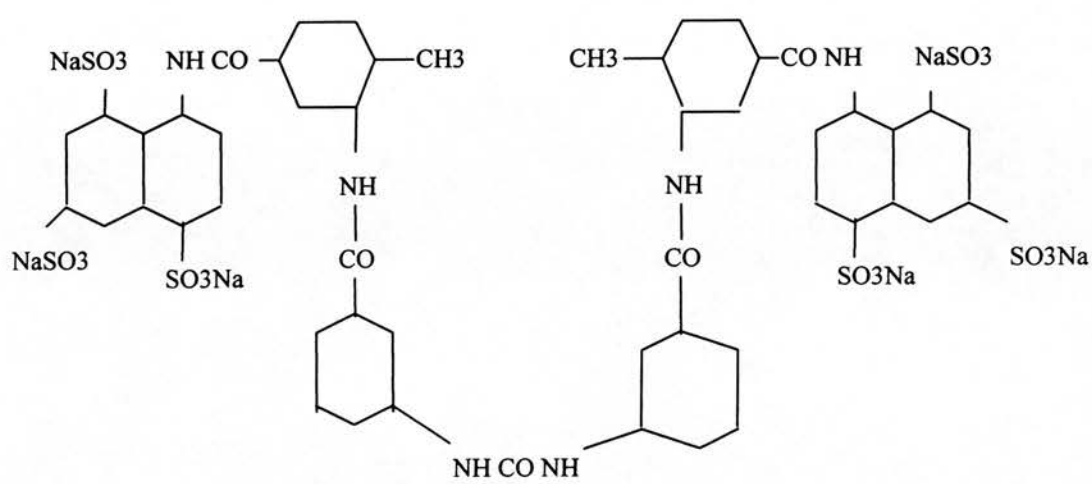
Figure 2.1: Molecular structure of suramin.

Despite its long clinical use the specific mode of action of suramin is poorly understood. Suramin is however, known to bind to albumin and light-density lipoprotein (LDL) in serum (Vansterkenburg *et al.*, 1993) that enter the trypanosomes primarily by receptor-mediated endocytosis of some of the serum proteins (Wang, 1995). Wang (1995) argued that since mammalian cells are also capable of performing receptor-mediated endocytosis of serum proteins, the preferential toxicity on trypanosomes exhibited by suramin must mean that either trypanosomes possess more vigorous endocytic activities on serum proteins in general or certain suramin-serum protein complexes are preferentially taken up by trypanosomes.

Suramin is known to be an inhibitor of many different dehydrogenases and kinases from mammalian, bacteria, and fungal sources. It has been found to inhibit dihydrofolate reductase, thymidine kinase and all the glycolytic enzymes in *T. brucei* (Chello and Jaffe, 1972; Wilson *et al.*, 1993). It is thought that suramin does not affect trypanosomes by inhibiting the function of any of the glycolytic enzymes inside the glycosomes. The genes encoding these enzymes are located in the nucleus, and the enzymes are synthesised on free polysomes in the cytoplasm and imported into the glycosomes posttranslationally without any proteolytic modification within 3-5 minutes (Hart *et al.*, 1987). It has been suggested that suramin possibly binds to the glycolytic enzymes in the cytoplasm within this short period and interferes with their import into the glycosomes (Wang, 1995). Since the glycolytic enzymes have an average half-life of about 48 hours inside the glycosome (Clayton, 1987) the inhibition of glycosomal protein import may lead to a gradual decrease of enzyme concentrations in the glycosome and a slowing down of energy metabolism in suramin-treated trypanosomes which has been observed in previous studies (Fairlamb and Bowman, 1980a; 1980b). This proposed mechanism of action of suramin has not been proven experimentally. However, it is considered that since resistance to suramin has not been a serious problem after over 70 years of use this may support the theory that suramin acts on multiple targets in trypanosomes (Wang, 1995).

2.5.3. Suramin complex

Following the demonstration that suramin formed a precipitable salt complex when mixed with pentamidine, thereby reducing the toxicity of the latter while preserving its prophylactic activity, suramin complexes were produced with almost all the available trypanocides (Williamson, 1970). A number of these complexes were shown to have long prophylactic activity in cattle. However, the problem of severe reaction at the site of injection, which often involved sloughing off of the drug depot limited their general use. Among these was the quinapyramine complex which was shown to be suitable for prophylaxis against *T. evansi* infection in horses in India (Gill and Malhotra, 1971).

2.5.4. Quinapyramine

Quinapyramine is a bis-quarternary salt synthesised from Surfen C molecule and belongs to the quinaldine group of trypanocides. It has been used either as quinapyramine dimethosulfate alone for therapeutic treatment or in combination with quinapyramine chloride for prophylaxis.

Quinapyramine sulfate is a 4-amino-6-(2-amino-6-methyl pyramid-4-ylamino)-2-methylquinoline-1-,1'dimetho (methyl sulfate); ($C_{19}H_{28}N_6O_6S_2$, molecular weight 532.6, Figure 2.2). The compound is readily soluble in water (1:2 at 20^0C), is active against *T. brucei, T. congolense*, and *T. vivax*. It is used for curative treatment at the recommended dose of 4.4 mg/kg, subcutaneously (Curd and Davey, 1950). Quinapyramine sulfate is well tolerated by cattle but some animals show post infection stress. Severe systemic reaction is observed in equines (Whiteside, 1960a). The toxic effects can be reduced by dividing the dose into two equal halves and administering the second half 5-6 hours later. Quinapyramine sulfate has marked prophylactic effect but this property was found to facilitate the development of drug-resistant organisms.

Quinapyramine chloride, 4-amino-6-(2 amino-6-methylpyrimid-4-ylamino)-2-methylquinoline-1-, 1'-dimethochloride dihydrate ($C_{17}H_{22}C_{12}N_6$, $2H_2O$, molecular

weight 417.3) is similar to the sulfate in physical appearance but it has very low solubility in water 1:850 at 20^0C).

It was shown that subcutaneous injection of the chloride resulted in the formation of a depot from which the drug was slowly released. However, blood concentrations were too low to effect cure of established infections. This led to the introduction of a mixture of the two compounds, quinapyramine sulfate (3 parts) and quinapyramine chloride (4 parts), marketed as Antrycide Prosalt® by ICI Ltd. UK, for prophylaxis. A revised formulation (RF) was later introduced which contained only half the quantity of quinapyramine chloride. This was found to be as effective as the original preparation, and at the same time produced less local tissue reactions. The RF also had an added effect on *T. simiae* in pigs.

Quinapyramine was used extensively for treatment against infections caused by *T. vivax, T. brucei, T. congolense* and *T. evansi* in all domestic animals. The Antrycide prosalt® has also been widely used, usually given every 2 months. (Unfortunately widespread resistance to quinapyramine (Williamson, 1970; Leach and Roberts, 1981; Losos, 1986) led to the withdrawal of quinapyramine from the market in many parts of Africa in the 1970s.) The drug was re-introduced in the market under two different names. Tribexin Prosalt® (quinapyramine sulfate:quinapyramine chloride, in the ratio of 3:4; Indian Drugs and Pharmaceuticals Ltd, Hyderabad, India) is recommended for use in *T. evansi* infections in donkeys and camels (Suryanarayana *et al.*, 1985). The other product, Trypacide® (May and Baker, UK), is available in two forms for field use. Trypacide sulfate is recommended for subcutaneous treatment of clinical cases, and Trypacide Pro-salt® (quinapyramine sulfate: quinapyramine chloride in the ratio of 3:2, May and Baker, UK) is recommended for prophylaxis (Kinabo, 1993).
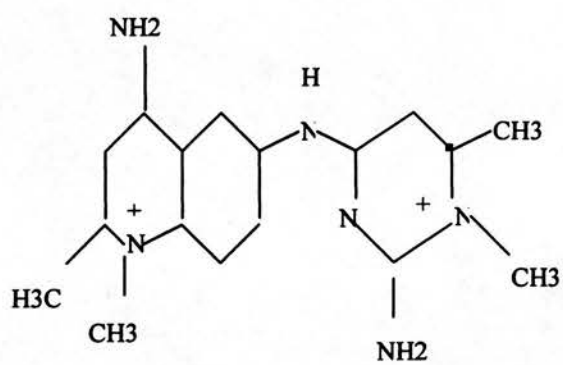
Figure 2.2: Molecular structure of quinapyramine

Due to the long pause in research following withdrawal of quinapyramine from the market not much information is available on the mechanisms of action of this drug (Kinabo 1993).

2.5.5. Isometamidium

Isometamidium (Samorin®, Trypamidium®) is a phenanthridinium compound whose antitrypanosomal activity was demonstrated over 5 decades ago (Kinabo 1993). The compound is similar to homidium except for an additional moiety of m-amidinophenyl-azo-amine (Wragg *et al.*, 1958) which is also part of the diamidine molecule (Figure 2.3). Isometamidium is thus thought to be a 'hybrid molecule' which exhibits some properties of homidium and diminazene.

Isometamidium is active against *T. congolense* and *T. vivax* in cattle. It is also of value against infections caused by *T. brucei* and *T. evansi* in donkeys, horses and camels (Zhang *et al.*, 1991; Kinabo, 1993). It is administered intramuscular at doses of 0.25 - 0.5 mg/kg for therapeutic purposes, and 0.5 - 1.0 mg/kg for prophylaxis. The maximum dose for resistant cases is 2.0 mg/kg.
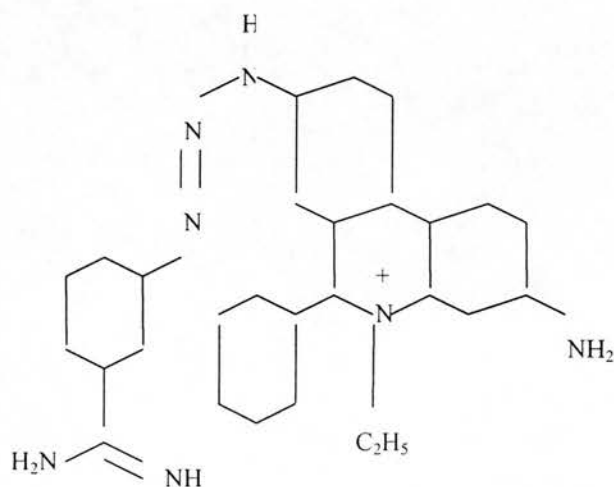
Figure 2.3: Molecular structure of isometamidium

Isometamidium is rapidly detected in plasma following intramuscular administration. Maximum plasma concentrations are attained within 1 hour following drug administration (Gilbert and Newton, 1982; Kratzer *et al.*, 1989; Kinabo *et al.*, 1991) and fall rapidly thereafter to low concentrations. Despite the rapid detection in the plasma the bioavailability of isometamidium is very low (Kinabo and McKellar, 1990). This is because the drug is bound at the injection site where it forms a primary depot from which it is slowly released to exert its prophylactic activity (Hill and McFadzean, 1963; Kinabo and Bogan, 1988a).

The mechanisms of action of phenanthridinium drugs to which isometamidium belongs is reviewed by Kinabo (1993). The primary mode of action of isometamidium is thought to be the blockage of nucleic acid synthesis through intercalation between DNA base pairs, inhibition of RNA polymerase, DNA polymerase and incorporation of nucleic acid precursors into DNA and RNA. Other biochemical reactions that have been suggested to contribute partly to their effects include modulation of glycoprotein biosynthesis, lipid membrane transport and, selective cleavage of kinetoplast DNA minicircles (Kinabo, 1993). Although the primary mechanism of action i.e. blockade of nucleic acid alone may not explain the basis of selective toxicity it has been suggested that there are a number of biochemical peculiarities demonstrated in trypanosomes that appear to be candidate targets for drug modulation which might explain the basis for selective toxicity. These include the cleavage of kinetoplast DNA minicircles, a unique peptide, trypanothione essential in maintaining the correct intracellular redox balance in trypanosomes (Fairlamb, 1990); a peculiar AMP binding protein and the novel glycosomes, organelles that contain a wide variety of enzymes involved in glycolysis, glycerol metabolism, CO_2 fixation, pyrimidine synthesis, purine salvage and ether lipid synthesis (Kinabo, 1993).

2.5.6. Diminazene aceturate

Diminazene aceturate (Berenil®, Farbwerke Hoechst AG), P,P'-diamidinodizoaminobenzene diaceturate tetrahydrate ($C_{22}H_{29}N_9O_6$. $4H_2O$; molecular weight 587.6; Molecular weight base = 281.2; Figure 2.4) was developed

chemically from Surfen C. It is readily soluble in water (1:14 at 20^0C). The drug is stable when dry but aqueous solutions are stable for only 2-3 days. Faircclough (1963) stated that the stability of Berenil$^®$ in solution has been increased by the addition of phenyl dimethyl pyrazolone (antipyrine) as a stabiliser, by the manufacturers.

Diminazene aceturate is the only member of the diamidine group used routinely for treatment of trypanosomosis in small animals. It is the commonest drug used against tsetse-transmitted trypanosomosis and is thought to qualify as a broad-spectrum trypanocide Losos (1986). The drug is effective in cattle, sheep, horses and dogs against *T. congolense* and *T. vivax* at 3-5 mg/kg, and against *T. brucei* at 5-7 mg/kg. It is not effective against *T. simiae*. Doses of 10 mg/kg have been found to be effective against *T. evansi* infection in cattle. The drug is administered subcutaneously. Diminazene aceturate is well tolerated in cattle (Fairclough, 1963), sheep, and man. However, it has been known to produce severe toxic reactions in camels at 7 mg/kg dose (Leach and Roberts, 1981), hence it is contraindicated. Low tolerance has also been observed in dogs in which it causes vascular injury manifested in brain damage (Losos, 1986). *T. evansi* infections have been shown to be fully susceptible to Diminazene and the drug also has activity against *T. rhodesiense*, and *T. gambiense*. Besides its trypanocidal effect, Diminazene has been shown to have high activity against *Babesia* infections, as well as having antibacterial and antifungal effects (Williamson, 1970; Leach and Roberts, 1981).

Diminazene aceturate is used primarily as a curative agent, and is extensively used against cattle trypanosomosis. It is rapidly excreted from the body. Almost all the drug is cleared through the kidneys within 24 hours following parenteral administration of a dose. This property has been important in lessening the risk of resistance development to the drug.
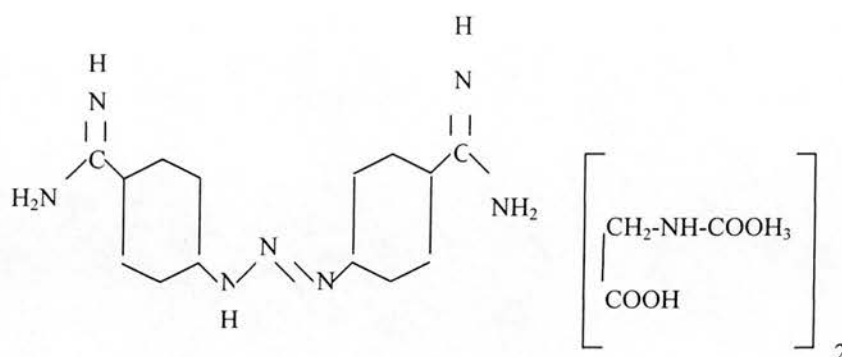
Figure 2.4: Molecular structure of diminazene diaceturate

Diminazene binds to specific sites in DNA via electrostatic and hydrogen bond forces (Hart *et al.*, 1987). It also inhibits the kinetoplast topoisomerase II in trypanosomes, resulting in cleavage of the minicircle DNAs. It is thought to interfere with RNA editing and RNA *trans*-splicing in trypanosomes (Wang, 1995).

Although resistance to diminazene in the field is not widespread quinapyramine and melarsomine appear to be able to induce cross-resistance to diminazene, both in the laboratory and in the field (Osman *et al.*, 1992). Preliminary studies suggest that the mechanism of resistance to diminazene is most likely the diminished uptake of the drug by resistant trypanosomes (Peregrine and Mamman, 1993).

2.5.7. Arsenicals

2.5.7.1.   *Drug evolution*

The use of inorganic arsenic in medicine dates back to the days of Hippocrates. Orpiment (arsenic trisulfide) and Fowler's Solution (potassium arsenite) were used as adjuncts to benzidine dyes in the early days of drug evolution. However, the introduction of the synthetic aromatic arsenical, Atoxyl in 1905 marked the turning point in the history of the therapeutic use of arsenic. This became the foundation of Ehrlich and Hata's work which eventually resulted in the development of arsphenamine (Salvarsan) in 1910 and later to tryparsamide both of which were effective against human trypanosomosis (Williamson, 1970).

The old arsenic preparations, however, continued to be used in the treatment of animal trypanosomosis. Williamson (1970) recorded that Livingstone in his missionary journey to Africa, used Fowler's Solution to treat a 'fly-struck mare' (victim of nagana); Lingard in 1893 showed that the arsonite solution together with cinchona alkaloids, had a therapeutic effect on the 'haematozoon' of surra, in India; and Bruce in 1896 also demonstrated that infection due to tsetse-borne trypanosomes were susceptible to treatment by arsenious oxide or sodium arsenite. Although arsenic treatment was practised in human infections its toxic reactions prevented its continued use in man.

Aliphatic compounds first prepared by Cadet in 1760 and characterised by Bunsen in 1837 (Williamson, 1970) were shown to be less toxic than inorganic arsenicals. However, due to their excretion through the lungs they imparted garlic-type odour to the breadth and were not acceptable. Atoxyl, which became the first acceptable organic arsenical was discovered incidentally as a by-product in the production of the triphenyl-methane dye, parafuscin. It was first thought to be a sodium salt of an anilide of arsenic acid, isolated by Be'champ in 1863. A number of studies that followed the use of Atoxyl led to the demonstration of its trypanocidal action by Thomas and Breinl in 1905 and later its true chemical nature by Ehrlich and Bertheim (Williamson, 1970).

Extensive use of Atoxyl in man brought to light its toxic effects on the optic nerve which caused optic atrophy and blindness, and new search for less toxic organic arsenicals continued. The result was the major discovery in 1910 of the arsenobenzene compound, Salvasan (arsenamine) which was active in syphilis; and an early precursor of tryparsamide, Arsenophenylglycine, was prepared. Tryparsamide (p-glycine amidophenylarsonate) was discovered during the 1914-1918 war and was first synthesised by the condensation of Atoxyl and chloroacetamide, by Jacobs and Heidelberger in 1922. It is a water-soluble salt which was found to be highly effective in human trypanosomosis, especially the late stage Gambian disease, for which it was the only available drug (Williamson, 1970). Unfortunately, optic atrophy and blindness still occurred in 5% of treated cases. Tryparsamide was used in combination with suramin between 1930-1940. Two newer drugs were later

introduced by Friedheim - melarsen in 1938 and melarsoprol (mel B) in 1946-1949 (Friedheim, 1949). Melarsoprol was obtained by reacting a toxic trivalent arsenical, melarsen oxide with British Antilewisite (BAL) (2,3-dimecapto propanol). The resulting compound both retained its trypanocidal activity and became significantly less toxic (Meshnick, 1984). Melarsoprol was found to be less toxic than tryparsamide, and because of its lipid solubility it is able to cross the blood-brain barrier. It has been used extensively for treatment of the late stages of human trypanosomosis. A water-soluble analogue of melarsoprol, mel W (trimelarsan) and an antimony-containing analogue of melarsoprol, Msb, have also been tested but offered no advantage over melarsoprol (Williamson, 1970; Meshnick, 1984).

2.5.7.2.   *Melarsenoxide cysteamine*

Melarsenoxide cysteamine (mel Cy, RM110, Cymelarsan®, Rhone Poulenc Toulouse, France), was discovered in 1985 as a member of the melaminyl thioarsonite group of compounds invented in the 1940s (Raynaud *et al.*, 1989a). The bis (amino-ethylthio)-4 melaminophenylarsenine dihydrohloride (Figure 2.5) is a water-soluble trivalent arsenical compound patented in 1985 (Friedhein), mainly for use in camels (Bujon, 1990). It is related to the drug mel B, the arsenical drug used for the treatment of late stage sleeping sickness in human trypanosomosis.

The introduction of mel Cy has been described as a clinical breakthrough in the chemotherapy of *T. evansi* infections in camels because the existing drugs have not been very much favoured in view of their high toxicity or lack of high efficacy (Ali *et al.*, 1985; Ali and Hassan, 1986). Mel Cy is effective primarily against infections caused by *T. evansi* in camels, cattle, horses and buffaloes (Sones *et al.*, 1989; Raynaud *et al.* 1989a; Lun *et al.* 1991).

Mel Cy is presented as a sterile white freeze-dried powder for injectable solutions. It is highly soluble in water. Mel Cy was used successfully to treat camels artificially infected with *T. evansi* (Tager-Kagan *et al.*, 1989). Subsequently, several workers have reported the successful treatment of various animal species, naturally or experimentally infected with *T. evansi* using mel Cy, including buffaloes (Lun, *et al.*, 1991), mice (Zweygarth and Kaminsky, 1990), cattle (Payne *et al.*, 1994a), camels

(Zelleke, *et al.*, 1989; Otsyula, *et al.*, 1992). Mel Cy is very effective against the *Trypanozoon* of the *T. brucei* group., namely *T. brucei, T. evansi* and *T. equiperdum*, in camels buffalo, goats and pigs, and *in vitro* (Lun *et al.* 1991; Otsyula, *et al.*, 1992; Zhang *et al.* 1992; Zweygarth and Kaminsky 1990; Zweygarth *et al.*, 1992), on *T. evansi* from horse, camel and cattle and *T. rhodesiense* and *T. gambiense* (Denning *et al.*, 1989).

Mel Cy has also been found to be effective against diminazene aceturate-resistant *T. brucei* (Zhang *et al.*, 1992; Zweygarth and Kaminsky, 1990) and stocks of *T. evansi* resistant to quinapyramine and suramin (Zweygarth and Kaminsky, 1990). Mel Cy was found to be 2.2 - 2.7 times more active against *in vitro T. brucei* stocks compared with a related, water-soluble arsenical, mel W (Trimelarsan) (Zweygarth and Kaminsky, 1990). In mg/kg mel Cy was more effective than other arsenicals (mel B and mel W) and suramin or diminazene on target parasites. It also seemed to be more effective than mel W on central nervous infections of *T. bucei* although with repeated daily treatment (Denning *et al.*, 1989). Mel Cy has been used as part of combination therapies in mouse models of secondary trypanosomosis (Jennings, 1993). It is not effective against *T. congolense* (Denning *et al.*, 1989). On *T. evansi* mel Cy is very quickly effective in early infections (low parasitaemia) or late infection (very high parasitaemia) in acute disease models as well as early or late stages of chronic disease models.

Mel Cy is a curative agent with no preventive or long acting effect (Denning *et al.*, 1989, Bujon, 1990). The recommended therapeutic dose in camels is 0.25-0.5 mg/kg administered subcutaneously or by deep intramuscular injection in the neck (Bujon, 1990; Sones *et al.*, 1989).

Studies of the pharmacokinetics of mel Cy have been reported by Baltz *et al.* (1989) and Raynaud *et al.* (1989b). Following administration a peak plasma concentration in cattle, horses and camels is achieved in about 15-30 minutes after which plasma levels decline rapidly. Absolute bioavailability in camels was 70-80% (Raynaud *et al.*, 1989b). In view of this short duration of trypanocidal level in plasma mel Cy has no prophylactic or long acting effect. The therapeutic index (lethal dose/therapeutic

dose) of greater than 10 (Raynaud *et al.*, 1989a) indicates that mel Cy is reasonably safe for use at the recommended dose. The drug has rapid trypanocidal activity and destroys trypanosomes within few hours. Complete recovery occurs rapidly following subcutaneous or intramuscular administration.

At recommended dose levels Cymelarsan has been shown to be well tolerated. After subcutaneous injection, mel Cy has been shown to induce only a transient local reaction (swelling and oedema) at the site of injection in cattle and camels which resolve after a few days (Kinabo, 1993). The drug has a wide margin of safety with maximum tolerated dose in the range of 3-4 mg/kg. It is safe for camels and has been recommended for use in pregnant animals (Bujon, 1990).
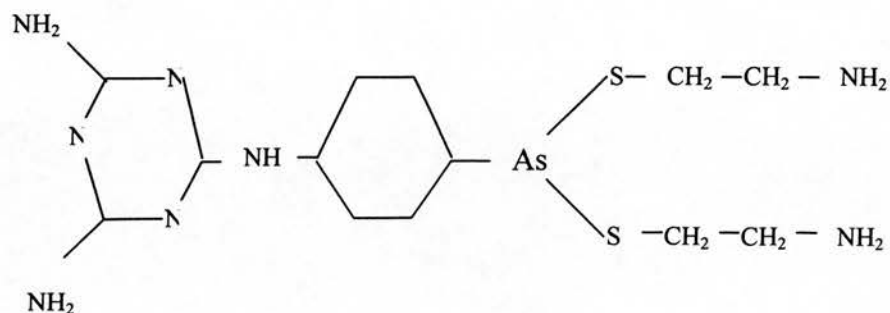
Figure 2.5: Molecular structure of melarsenoxide cysteamine (mel Cy)

The molecular events underlining the action of mel Cy have not been well characterised. It has been suggested that they are similar to those of other arsenical compounds such as melarsoprol. These compounds are thought to act primarily in two ways. The first mechanism is by interference with the energy generation processes through inhibition of pyruvate kinase (Flynn and Bowman 1974). The second mechanism is by interaction with trypanothione (Fairlamb, 1990). Mel Cy is thought to inhibit the metabolism of the cell by inhibition of trypanothione reductase, an enzyme which is found to be absent in the host (mammalian cells) but which is central to the regulation of the thiol/disulfide redox balance in the trypanosome.

2.6. CHEMOTHERAPY AND IMMUNE RESPONSE

This subject has been extensively reviewed by Doenhoff *et al.* (1991). The concept of a combined action between drugs and the immune response was started by Paul Ehrlich (1907). McDonah (1920) raised the possibility that antimony acted on parasites in association with the protein particles in serum, and York (1925) reported that antimalarial chemotherapy was likely to be more effective after patients had a chance to acquire some immunity (reviewed in Doenhoff, et al., 1991). The

relationship between drug and the immune response however was explored experimentally between 1948-1949, when Taliaferro (1948), found that acquired immunity was involved in the cure of malaria in chickens by quinine.

At present it is known that immunosuppression reduces the efficacy of experimental chemotherapy of several parasitic diseases, including schistosomiasis (Sabah *et al.*, 1985; Brindley and Sher, 1987; Fallon *et al.*, 1996), malaria (Lwin *et al.* 1987), trypanosomosis (De Gee *et al.*, 1983; Bitonti *et al.*, 1986), and onchocerciasis caused by *Onchocerca lienalis* (Bianco *et al.*, 1986). Greater cure rates have been achieved in immunosuppressed hosts infected with schistosomiasis and malaria by the simultaneous administration of immune serum or immunoglobulin with a drug. This further substantiates the existence of a close relationship between the immune response and chemotherapy (Doenhoff *et al*, 1991).

The mechanisms of immune dependent chemotherapy has been extensively studied in schistosomiasis. It is established that four schistosomal drugs (antimony, oxamniquine, hycanthone and praziquantel) kill fewer (15-56% less) *Schistosoma* adult worms in immunosuppressed mice than in immunologically intact control animals. But the actions of two drugs niridazole and amoscanate, were independent of the immune response (Sabah *et al.*, 1985).

The efficacy of antimony, oxamniquine and praziquantel is enhanced by passive transfer of immune serum simultaneously with drug administration in *S. mansoni* infected mice, indicating the role of humoral immune effector mechanisms in this phenomenon. Administration of serum alone does not have any deleterious effects on the survival of adult *S. mansoni* confirming that the immune serum acts synergistically with the drug (Lambertucci *et al.*, 1989; Doenhoff *et al.*, 1991).

Praziquantel for example, is thought to act by destabilising the surface lipid membranes of *S. mansoni*. One of the early morphological changes is the disruption of the membrane over the tubercles on the dorsal surface of male *S. mansoni*, and an increase in parasite-specific antigenicity is detectable after treatment of the worms *in vitro* (Harnett and Kusel, 1986). It has Been shown that antisera that kill worms

synergistically with praziquantel, *in vivo* react particularly with the tubercles of drug-treated male worms (Brindley and Sher, 1987).

The two antigens specifically implicated as targets of synergistically active antibody in immune-dependent chemotherapy of *S. mansoni* are a 27 kDa polypeptide with esterase activity, and a 200 kDa glycoprotein. Both these antigens are located on or near the surface of the membranes but the epitopes relevant to antibody-assisted worm killing by praziquantel normally remains unexposed until drug treatment. It is suggested that membrane attachment may be a requirement for an antigen's involvement in immune-dependent chemotherapy but this has not been experimentally verified (Doenhoff *et al.*, 1991).

A second school of thought suggests that antibodies may not act exclusive of cellular effector mechanisms in immune-dependent chemotherapy. The drug Oltipraz has been found to enhance cytotoxic effects of neutrophils against *S. mansoni* adults *in vitro* (Mkoji *et al.*, 1990). Rapid cellular infiltrations of worms occurring after praziquantel treatment *in vivo* have been thought to be a manifestation of cell mediated immune cytotoxicity that is dependent on prior drug-induced damage to the parasite (Doenhoff *et al.*, 1991).

In leishmaniasis pentavalent antimony therapy was found to be less effective against *Leishmania donovani* in infected T-cell-deprived and glucocorticoid-treated mice when compared with mice with intact immune responses. Furthermore, improved results have been obtained from experimental and clinical treatment of *L. donovani* with a combination of antimony and gamma interferon. It is suggested that the cytokine enhanced the rate of intracellular killing of the parasite, thus controlling the infection (Doenhoff *et al.*, 1991).

Studies on trypanosomes have shown that in addition to trypanosomes which are sequestrated from the drugs in cryptic sites and those which exhibit drug resistance, trypanosomes can proliferate following drug treatment owing to a dysfunction in normal immune responses. The curative effects of a number of trypanocidal compounds have been found to be reduced in splenectomised animals suggesting that a decrease in phagocytic activity influences drug efficacy. De Gee *et al.* (1983) and

Bitonti *et al.* (1986) demonstrated that antibody specifically that directed against the variant specific antigen, potentiates the effectiveness of difluoromethylornithine (DFMO). Gilbert and Newton (1982) suggested that the efficacy of ethidium bromide in curing cattle is dependent on an efficient antibody response. Frommel (1988) also demonstrated that immnuosuppresssion effectively decreases the efficacy of mel B treatment in mice.

The phenomenon of immune-dependent chemotherapy has obvious implication on the development of drug resistance. This was earlier demonstrated by Carter *et al.* (1973). These workers found that the treatment of a transplantable mouse lymphoma with asparaginase achieved a permanent cure in immunologically intact mice but not in T-cell-deprived mice. These mice eventually died and their recurrent tumours were found to be solidly asparaginase resistant. It was concluded that although the main effects of the drug was the destruction of sensitive tumour cells, there was a small residual of asparaginase-resistant cells that was destroyed by the host immune response. In immunosuppressed animals, the drug-resistant tumour cells survived and grew back to kill the host.

The interpretation of these results have been applied to many other situations involving infections and drug treatment, especially infections involving rapid replicative potentials. Frommel (1988) demonstrated that treatment of immunosuppressed animals with a mel B dose that is curative in immunocompetent animals results in the selection of drug-resistant trypanosomes.

This concept has now formed the basis for the selection of drug-resistant trypanosomes using immunosuppressed laboratory hosts (Osman *et al.*, 1992; Mutugi, 1993).

2.7 DRUG RESISTANCE

2.7.1. Definitions

2.7.1.1.   *Resistance*

Resistance is defined as the ability of an organism to remain unaffected by noxious agents such as poisons, toxins, irritants, or pathogenic micro-organisms in its environment (Harinasuta and Bunnag, 1988). For the most part resistance is a genetic endowment of an entire species with respect to a particular agent. It can be absolute, in which case all members of the species are insusceptible to a specific agent. If resistance is relative, racial and individual differences occur within the species, one race or member being more resistant than the other (Harinasuta and Bunnag, 1988). In the context of parasitology and trypanosomosis in particular two major types of resistance are important: host resistance and parasite resistance. This thesis is mainly concerned with resistance of parasites to drugs, and specifically resistance of trypanosomes to trypanocides.

2.7.1.2.   *Drug resistance*

Drug resistance has been defined in different ways. In malaria drug resistance was defined as the ability of a parasite to multiply or to survive in the presence of concentrations of a drug that normally destroy parasites of the same species or prevent their multiplication.   Such resistance may be relative (i.e. yielding to increased doses of the drug tolerated by the host) or complete (withstanding maximum doses tolerated by the host (WHO, 1963). Drug resistance has also been defined as a significant increase in the ability of individual parasites within a strain to tolerate doses of the compound which would be lethal to the majority of parasites in a   . normal population of the same species (Harinasuta and Bunnag, 1988). A decrease in sensitivity may be caused by non-genetic or genetic changes in the target cell. If a change in sensitivity does not involve a change in chromosomal or extrachromosomal DNA it is thought to be the result of phenotypic adaptation. This may take place in culture after a long exposure to the drug under conditions

favourable for the cell or organism. Phenotypic resistance is not stable and is usually lost upon transfer to a drug-free medium. This instability, however, is to be distinguished from regression of resistance which may be due to reduced fitness of genetic mutants (Harinasuta and Bunnag, 1988).

Geary *et al.* (1986) described drug resistance as a stable genotypic variant, selected from a normally sensitive population by drug exposure, that has the ability to survive and multiply in the presence of normally effective drug concentrations.

2.7.1.3. Drug failure

Drug failure describes a situation where by a drug, administered in the appropriate dosage does not produce the recognised action against the target parasite for the reasons that not enough of the drug or its active metabolite has reached the parasite (Harinasuta and Bunnag, 1988). In contrast in a situation of drug resistance, the drug has reached the parasite but the parasite has become adapted to the introduced chemical environment and survives.

2.7.2. Drug resistance in parasites

2.7.2.1   *Plasmodium falciparum*

A report of the distribution of chloroquine-resistant *Plasmodium falciparum* malaria (Harinasuta and Bunnag, 1988) suggests that resistance is widespread in countries all over the world where *P. falciparum* malaria is endemic. Sulfadoxine/pyrimethamine (S/P) introduced in the 1960s as the first line alternative antimalaria drug combination suffered tremendous decline in cure rates, and there was need for use of alternative drugs. Quinine, an old antimalarial drug regained its value because of increasing occurrence of resistance to synthetic antimalarials (Harinasuta *et al.*, 1965). There have been reports in various parts of the tropics of reduction in efficacy of Quinine (Harinasuta and Bunnag, 1988). Cross-resistance between chloroquine and quinine has been observed in some strains of *P. falciparum* (Harinasuta and Bunnag, 1988). Cases of primary resistance to mefloquine have also been reported in Thailand and East Africa and primary resistance to combination of mefloquine with

sufadoxine and pyrimethamine (MSP) has been reported in Indonesia (Harinasuta and Bunnag, 1988).

2.7.2.2.   *Plasmodium vivax*

Widespread resistance of *Plasmodium vivax* to pyrimethamine has been reported (Harinasuta *et al.*, 1985) and cross-resistance between pyrimethamine and proguanil has also been observed. The tropical strain of *P. vivax* has been reported to be particularly resistant to primaquine, an antirelapse drug used against the subtropical strain of *P. vivax* (Harinasuta and Bunnag, 1988).

2.7.2.3.   Other protozoa

Similar problems of drug resistance has been encountered in other protozoans. The most commonly used drug for the treatment of amoebiasis and giardiasis have been the 5-nitroimidazole derivatives such as metronidazole, tinidazole and secnidazole. Metronidazole was first introduced for the treatment of *Trichomonas vaginalis* infection and later intestinal amoebiasis. Relative resistance of *T. vaginalis* infection to metronidazole and *Giardia lamblia* to quinacrine or metronidazole following treatment has been reported (Harinasuta and Bunnag, 1988).

2.7.2.4.   Helminths

Anthelmintic resistance of livestock helminths especially gastrointestinal nematodes has constituted a serious problem to livestock industry in many countries (Kelly and Hall, 1979; Geerts *et al.*, 1997). Resistance to anthelmintics of nematodes in sheep, goats and horses have been reported mainly from countries such as Australia, South Africa, Netherland, New Zealand and countries of South America, where anthelmintics have been used at high frequency. These include benzimidazole resistance in *Haemonchus spp*, *Ostertagia spp*, *Cooperia spp Teladorsagia spp* and *Trichostrongylus spp* (Borgsteed *et al.*, 1996; Geerts *et al.*, 1997).

2.7.3. Drug resistance in trypanosomosis

2.7.3.1. *Development of drug resistance*

The most common causes of the development of drug resistance in trypanosomosis in the field include underdosing of drug due to incorrect estimations of animal body weight, especially in situations of mass treatment (Whiteside, 1961). Irregular use of prophylactic and therapeutic drugs is another factor that favours the emergence of drug-resistant trypanosomes. This occurs especially where there is high tsetse fly challenge and leads first to 'individual' resistance and subsequently to 'areal' resistance (Raether, 1988). Prophylactic drugs are known to induce resistance more rapidly in trypanosomes than do therapeutic drugs. Therapeutic drugs reach trypanocidal plasma levels relatively quickly and are believed to be rapidly metabolised and excreted from the body. Prophylactic drugs, however, take a longer time to be completely excreted from the body. Thus in areas with high incidence of infection subcurative drug levels may already exist towards the end of protection period. Repeat treatment is usually required to restore trypanocidal plasma concentrations.

2.7.3.2. *Natural resistance*

Natural resistance has also been identified in trypanosomosis. Natural drug resistance refers to the variation in drug sensitivity that is not dependent on previous exposure to the drug concerned. This phenomenon has been demonstrated in *T. vivax* and *T. congolense*. Strains of *T. vivax* from West Africa were found to be more susceptible to homidium than were *T. congolense* strains from East Africa (Scott and Pegram, 1974). In contrast, Williamson (1970) reported that *T. congolense* strains appeared to be more susceptible to diminazene than were *T. vivax*. This varying intrinsic sensitivity of these species was thought to explain the initial appearance of homidium- resistant *T. congolense* strains and diminazene-resistant *T. vivax* strains (Jones-Davies, 1967a, 1967b). Such variation could however be as a result of persistent cross-resistance induced by other drugs used previously.

Differences in drug sensitivities of stocks of the subgenus *Trypanozoon* have also been reported. Dukes (1984) demonstrated that West African stocks of *T. brucei* were not as sensitive to pentamidine and diminazene as typical East African stocks.

2.7.3.3.   *Cross-resistance*

Cross-resistance refers to resistance to a drug which has developed as a result of previous exposure of trypanosomes to a different drug of the same series or even to a drug of unrelated series. The occurrence of trypanosomes showing cross-resistance to several drugs has been well documented and is thought to be due to close relationship between compounds (Whiteside, 1961; Williamson, 1970). Multiple drug resistance has also been reported in a population of *T. vivax* in Kenya which proved resistant to all available drugs including isometamidium chloride, diminazene aceturate, homidium chloride and quinapyramine sulfate (Rottcher and Schillinger, 1985).

The widespread cross-resistance in trypanosomes to the few available trypanocides has constituted one of the greatest problems in the use of both chemotherapy and chemoprophylaxis in the control of animal trypanosomosis. Cross-resistance can only be effectively controlled by using drugs which do not induce resistance to each other and which can be used alternatively in the field when resistance to either drug appears. The concept of "sanative" pairs of drugs was proposed by Whiteside (1960b) after observation of the cross-resistance behaviour of trypanosomes to prophylactic and curative drugs. These drug pairs include homidium /diminazene and isometamidium /diminazene, which show no cross-resistance. In practice, for curative programmes homidium is used until evidence of resistance appears then it is replaced with diminazene which controls infections in cattle reinfected with homidium-resistant trypanosomes, and homidium is used again after a period of one year or more (Williamson, 1970). Isometamidium and diminazene are recommended for use in prophylactic field programmes. Usually quarterly prophylactic injections with isometamidium were supplemented by block treatment with diminazene at regular intervals, such as every 6 months prior to routine treatment with isometamidium (Williamson, 1970). Boyt (1985), however observed that the

sanative drug diminazene will not control the situation if the challenge becomes too high as a result of increasing rates of reinfection with resistant trypanosomes.

2.7.4. Stability of resistance to trypanocides

Observations on the stability of drug resistance in trypanosomes undergoing cyclical transmission have been contradictory. Some observations (Whiteside 1962; Jones-Davis, 1968) suggest that drug resistance is stable and transmissible, while other investigators have assumed that drug resistance in a trypanosome population is transient in the absence of drug pressure and infected animals. In a series of experiments Gray and Roberts (1971a, 1971b) found that resistance to curative doses of trypanocides was of a stable nature in *T. vivax* and *T. congolense*, while the parasites were transmitted through tsetse and cattle. However, it was assumed that, under field conditions competition between resistant and sensitive parasites in the trypanosome population may lead to an advantage to the sensitive forms resulting in a gradual disappearance of drug-resistant parasites. Similar studies (Osman *et al.*, 1992; Mutugi, 1993; Scott *et al.*, 1996) suggests that resistance in trypanosomes induced experimentally in either immunosuppressed or non-immunosuppressed animals was stable when transmitted to non-immunosuppressed hosts, either via syringe passage or cyclically through tsetse fly.

2.7.5. Relapse infections

The commonly used trypanocides such as diminazene, isometamidium and ethidium, do not cross the blood-brain barrier or produce constant trypanocidal concentrations in body cavity fluids and intracellular tissues (Luckins and Gray, 1979). In chronic infections of *T. brucei* patent parasitaemia after drug therapy could occur because of the appearance of parasite populations from such privileged sites as the cerebrospinal fluid and/or intracellular tissue spaces. Similar relapse infections arising from intracellular tissue spaces have also been found to occur in *T. congolense* infections (Raether, 1988).

Melarsoprol and related arsenical drugs are able to cross the blood-brain barrier because of their lipid solubility and hence are very useful in the treatment of late

human trypanosomosis in which the central nervous system is involved. It has been found that in chronic infections of *T. brucei* relapses may occur if treatment was started too late because drug sensitivity changes as the infections progress. Complete cure is obtained when drugs are given in the early stage of infection. Furthermore, in late stage (*T. brucei*) infections with CNS involvement treatment with non-arsenical drugs only results to apparent cure. This is because the parasites only disappear from circulation but after a period of weeks they r -establish themselves in the circulation (Jennings *et al.*, 1979; Whitelaw *et al.*, 1985).

2.8. MECHANISMS OF DRUG RESISTANCE

It is recognised that drug resistance can be heritable which means its development may involve changes (mutations) in the genetic material (DNA) which can be passed from one generation to the other (Geary *et al.*, 1986). Drug resistance can also be caused by non genetic changes in the target cell as a result of phenotypic adaptation following prolonged exposure to drug under conditions favourable to the cell (Georgopopulos, 1982). Such phenotypic resistance is not stable and is lost upon transfer to a drug free medium. When cells or organisms which exhibit these mutations are exposed to the action of a drug against which the resistance is developed they are at a selective advantage over the cells without the mutation. As a result the drug destroys the non mutant cells while the cells with the mutations survive and reproduce. With continuous drug pressure this results in a gradual build up of a resistant population.

There are differences in opinion as to the actual time point when drug resistant mutants arise i.e. whether they arise because of the presence of the drug or whether the drug only selects pre-existing mutants. The occurrence of resistance by both processes has, however, been recognised. For example variations in drug sensitivity that is not dependent on previous exposure to the drug concerned, has been demonstrated in *T. vivax* to homidium (Scott and Pegram, 1974) and *T. congolense* to diminazene (Williamson, 1970). It is also recognised that such resistance could be a result of cross resistance induced by other drugs.

Extensive work on drug resistance has mainly focused on diseases which are of medical importance and only recently has attention been drawn to the study of mechanisms of drug resistance involving diseases affecting animals. Different proposals have been put forward as possible mechanisms by which pathogens evade the actions of drugs.

2.8.1. P-glycoproteins and multidrug resistance (mdr)

The basis of what is presently called multidrug resistance was provided by the work of Paterson and Briedler, and Juliano and Ling in the 70s (reviewed in Broxtermann *et al* (1995). These Scientists selected Chinese hamster cells for resistance to cytotoxic agents, dactinomycin and colchicine, respectively, and obtained cells that were cross resistant to a broad group of drugs. They also found the expression of a 170 kDa plasma membrane protein associated with the resistant phenotype, a protein which is now known as P-glycoprotein (Pgp) (Broxtermann *et al.*, 1995).

Multidrug resistance (MDR) phenotype refers to cross resistance to several structurally and functionally unrelated drugs which have different targets. Typically it is found in antitumor drugs such as anthracyclines, vinca alkaloids, actinomycin D, all of which have different targets. Cross resistance to such a wide variety of agents has been thought to be caused by a decrease in drug concentration at the intracellular target sites. This could be due to decrease in drug uptake, enhanced drug efflux, or altered metabolism resulting in increased detoxification of the drug in the cell (Broxtermann *et al.* , 1995).

MDR has been found to be present in normal mammalian tissue. However its real function is not well known. It is suggested that it acts as an ATP-dependent transporter in the liver, and has a role as a volume-regulated chloride channel. Low levels of mouse MDR mRNA have been found in the kidney, placenta, heart and adrenals, and the highest levels can be found in the endometrial glands of the uterus during pregnancy. The mechanisms of activation of the MDR include mutation, mRNA level changes, gene amplification or a combination of them (Upcroft, 1994). The MDR gene has been cloned and identified in humans (MDR1) and its protein

product P-glycoprotein (Pgp) has been identified as a drug transporter molecule, causing drug transport out of cells (Broxtermann *et al.* , 1995).

The P-glycoproteins are large membrane proteins found in the plasma membrane of cells that can function as energy-dependent extrusion pumps by forming channels across the lipid bilayer of the plasma membrane. These confer resistance to cytotoxic drugs by an active drug efflux system which removes a drug from cells so actively that it does not have an oppoturnity to interact with its cellular target (Ouellette and Papadopoulou, 1993). Although P-glycoproteins are found in normal, drug sensitive cells amplification or over-expression of the genes that code for these proteins have been found to be a consistent feature in many drug resistant cells, such as cancer cells, bacteria, yeast and protozoan parasites (Ouellette and Papadopoulou, 1993; Upcroft, 1994).

Calcium channel blockers such as verapamil are known to reverse multidrug resistance in mammalian and other cells (Ouellette and Papadopoulou, 1993).

2.8.2. Gene amplification

Gene amplification has been shown to be an important step in the development of drug resistance *in vivo* in *Leishmania* species, and many drug resistant mammalian cells (Segovia, 1994). The mechanism of resistance involves the overproduction of the target gene product mediated by an increase in copy number, and consequently, in RNA being expressed from the target enzyme gene (Segovia, 1994; Gueiros-Filho *et al.*, 1995).

Antifolates, such as methotrexate, are widely used as antitumor and antimicrobial agents. These are inhibitors of dihydrofolate reductase (DHFR) which reduces dihydrofolate to tetrahydrofolate which is essential for DNA synthesis. In parasitic protozoa DHFR is encoded in a fusion polypeptide that contains the enzyme thymidylate synthetase (TS). Overexpression of genes as a consequence of gene amplification has been reported in methotrexate-resistant *Leishmania major* (Segovia, 1994). Such lines show elevated activity of DHFR and the amplification of the structural bifunctional dhf-ts. Similar amplifications have been shown in *L.*

mexicana made resistant to tunicamycin, an inhibitor of N-acetylglucosamine -1-phosphate transferase (NAGT); and other species of *Leishmania* resistant to oxianions and difluoromethylornithine (DFMO) (Foote and Cowman, 1994). Resistance of *Plasmodium* species to antifolates has also been associated with overproduction of the target enzyme, DHFR (Foote and Cowman, 1994).

2.8.3. Alteration of drug target

This involves alteration of the drug target in such a way that the drug is not able to bind. This mechanism of resistance has been identified in bacteria such as *Streptococcus* and *Staphylococcus* species. These alter their ribosomal structure to become resistant to sulphonamides and tetracyclines (Bauernfeind and Georgopapadakou, 1995). Also resistance due to alterations in an enzyme resulting from point mutations in a gene that codes for that enzyme has been demonstrated in *Leishmania* species (Segovia, 1994). Similar alterations in dihydropteroate synthetase enzyme has been shown to be involved in the mechanism of resistance to sulpha drugs in *P. falciparum* (Foote and Cowman, 1994).

2.8.4. Activation or development of alternative metabolic pathway

Resistance of gram negative bacteria to sulfonomides has been found to result from the activation of an enzymatic bypass in the folic acid pathway (Bauernfeind and Georgopapadakou, 1995).

2.8.5. Enzymatic inactivation or modification of drug

This forms an important mechanism of resistance to antibiotics such as the production of β-lactamase against the β-lactams or production of penicillinase by some bacteria.

2.8.6. Decreased intracellular accumulation

An important mechanism of resistance involve preventing a drug from reaching its intracellular target. This could occur through altered drug transport or enzymatic inactivation. Altered drug transport has been found to involve either decreased

uptake (influx) or increased efflux of the drug. An increased drug efflux involves an overefficient pump-out mechanism that pumps drug out almost as rapidly as it is taken in such that the drug does not attain high enough concentration to be toxic to the cell. This may involve the P-glycoproteins as observed in multidrug resistance (see section 2.8.1) or related systems which are not exactly P-glycoproteins. The latter are found in bacteria resistant to oxianions such as arsenite or antimony (Ouellette and Papadopoulou, 1993).

Most studies on drug resistance indicate that there is decreased accumulation in resistant parasites. It has been shown that uptake of sulpha drugs is reduced in sulphadoxine-resistant *P. falciparum* (Foote and Cowman, 1994). Similarly chloroquine-resistant *P. falciparum* are found to accumulate less chloroquine than their sensitive counterparts. It is not fully established whether the reduced accumulation of chloroquine is due to reduced uptake or enhanced efflux (Ward *et al.*, 1995). However biochemical and genetic evidence to the existence of an efflux process have been reported (Krogstad *et al.*, 1987). Verapamil, a classic reversal of multidrug resistance in cancer cells, have been shown to increase chemosensitivity to chloroquine in resistant isolates of *P. falciparum*. Ward *et al.* (1995) demonstrated the existence of two phenotypically distinct resistance mechanisms in highly chloroquine-resistant *P. falciparum* - one sensitive to verapamil and the other insensitive. The verapamil sensitive component was suggested to be the component which is modified in response to drug pressure.

Several *Leishmania* cell lines have been shown to be resistant to methotrexate because of decreased uptake of the drug. This has been attributed to mutations in the high affinity folate carrier, since methotrexate, an antifolate, is an DHFR inhibitor (Segovia, 1994; Ouellette and Papadopoulou, 1993)

In trypanosomes decreased uptake of pentamidine was reported in *T. brucei* (Damper and Patton, 1976; Berger *et al.*, 1995). Reduced accumulation of radiolabelled drug was associated with resistance to isometamidium (Sutherland *et al.*, 1991, 1992a). A mathematical model, however, failed to demonstrate that reduced accumulation was due to reduced uptake of the drug (Sutherland *et al.*, 1992b). More recent work

suggest that resistance to melaminophenyl arsenicals in *T. brucei* (Carter and Fairlamb, 1993; Scott *et al.*, 1997) and diamidines in *T. equiperdum* and *T. brucei* (Barrett *et al.*, 1995; Carter *et al.*, 1996) involved reduced accumulation as a result of reduced uptake .

The work presented here was an attempt to further investigate into the mechanisms of resistance to melaminophenyl arsenicals in trypanosomes by the exploration of drug transport mechanisms in both the drug sensitive and drug resistant *T. evansi*.

CHAPTER THREE

GENERAL MATERIALS AND METHODS

3.1. EXPERIMENTAL ANIMALS

All animals used in this study consist of adult female Tyler's original (TO) mice 20-30 gm. Mice were housed in approved facilities and fed mice cubes and water *ad libitum*.

3.2. TRYPANOSOMES

Studies were carried out on an Indonesian stock of *T. evansi* which had been cloned and maintained at the CTVM as TREU 1840. Other details of isolation of this isolate were not available. TREU 1840 was used to make drug resistant lines.

3.3. ANIMAL INFECTIONS

Trypanosomes were grown in mice for use in experiments requiring *in vivo*-derived trypanosomes. Depending on numbers of trypanosomes required for any experiments one or more mice were infected with a particular stabilate. Mice were injected intraperitoneally (i.p.) with 0.2 ml per mouse of frozen blood stabilate. For drug-resistant trypanosome stocks only original stabilates (unpassaged) were used for mice infections. However for the drug-sensitive stock up to three passages away from the original stabilate were used. In this case 0.2 ml of blood from an infected mouse was transferred to each of a new set of mice. Parasitaemia was monitored by microscopic examination of wet mount preparations of tail blood. Levels of parasitaemia were determined by visual estimation of the approximate number of trypanosomes per microscope field at x40 objective, according to the method of Herbert and Lumsden (1976). At parasitaemias of approximately 5×10^8/ml infected mice were anaesthetised using halothane and bled terminally by cardiac puncture using heparin as anticoagulant at final concentration of 5 IU/ml.

3.4. SEPARATION OF TRYPANOSOMES FROM INFECTED MOUSE BLOOD

For use in *in vitro* assays requiring *in vivo*-maintained trypanosome lines trypanosomes were separated from infected mouse blood by anion exchange chromatography using DEAE cellulose columns (Lanham and Godfrey, 1970). DEAE-cellulose (Whatman, England, DE$_{52}$), 500 gm, was suspended in 2 litres of phosphate buffered saline (PS pH 8.0). The main bulk of the exchanger was allowed to settle and the supernatant fluid containing the fines was removed. The DEAE cellulose was washed again by decantation with 2 litres of PS solution. The pH was adjusted to 8.0 using 5% orthophosphoric acid. The DEAE cellulose was further washed 2 times after pH adjustment by decantation with 2 litres of PS solution. It was then resuspended in fresh PS solution and sterilised by autoclaving. The DEAE cellulose was stored at 4°C.

For separation of trypanosomes from 1-3 ml of blood the DEAE cellulose was gently shaken to resuspend. Columns consisting of 60 ml plastic syringes were previously prepared, plugged with approximately 5 gm of glass wool, sealed in autoclave bags and autoclaved. Phosphate buffered saline solution containing 1% (w/v) glucose (PSG, pH 8.0) was also previously prepared, filter sterilised using 0.22 µm acetate membrane and stored at 4°C. The column was asceptically opened and mounted on a burette stand. The glass wool inside the column was soaked by running some PSG through the column. The column was packed carefully with successive volumes of the DEAE cellulose slurry up to a height of 7-10 cm. The adsorbent was equilibrated with 4 times volume of PSG which was allowed to elute. When the surface of the adsorbent was firm 1-3 ml of heparinized infected blood was applied at the top and allowed to enter into the adsorbent cellulose. PSG was run through the column by addition of drops using a 5 ml plastic pipette until about 20-40 ml of eluate was collected. The appearance of trypanosomes in the eluate was monitored by microscopic examination of drops of eluate on a microscope slide using phase contrast microscope. Trypanosomes in eluate were collected in universal bottles on ice. The eluate was centrifuged at 1000x g for 10 minutes to concentrate the trypanosomes.

3.5. PREPARATION OF TRYPANOSOMA EVANSI WHOLE BLOOD STABILATES FOR CRYOPRESERVATION

Five to ten mice (depending on the number of stabilates required) were each injected i.p., with 0.2 ml per mouse of blood of the appropriate trypanosome stabilate. At high parasitaemia mice were bled under anaesthesia in 5 IU/ml final concentration of heparin. The blood was pooled in a graduated centrifuge tube partially embedded in ice. An equal volume of freshly prepared 15% dimethyl sulfoxide (DMSO, SIGMA) in PSG was added to the blood and mixed thoroughly using a Pasteur pipette. The blood/DMSO mixture was dispensed in aliquots of 0.5-1.0 ml into cryopreservation tubes and capped. The tubes were first placed in the vapour phase of liquid nitrogen for at least 4 hours or overnight. They were then transferred into the main liquid nitrogen bank where they were stored at -181°C. Record cards containing the details of stabilates were completed.

3.6. CLONING OF TRYPANOSOMES IN VIVO

In vivo-derived drug-resistant trypanosomes were cloned in immunosuppressed mice according to the method of Smith *et al* (1982). Eight to ten mice were treated intraperitoneally with cyclophosphamide at 300 mg/kg body weight, 24 hours prior to injection with trypanosomes. A frozen stabilate of the appropriate trypanosome stock was removed from liquid nitrogen, thawed quickly by rubbing in between palms and 0.2 ml of the parasitaemic blood injected intraperitoneal into normal mouse. Trypanosomes were grown for 2-3 days. When parasitaemia was in exponential growth phase 10 μl of blood was taken aseptically from the snipped tail of the infected mouse using a 20 μl Finn pipette and tip, and diluted in 90 μl of PSG/1% normal mouse serum mixture on ice. Ten-fold serial dilutions of trypanosome suspensions in PSG/1% mouse serum mixture were made. The dilutions were examined microscopically and a dilution that gave a 0-1 trypanosome per "microdrop" was found. This dilution was used for cloning.

To isolate a single trypanosome a microdrop (less than 0.2 μl) of the selected trypanosome dilution was taken using a 0.2 μl pipette and tip and placed on a

chamber of a piece of glass which was marked into square areas. The microdrop was tiny enough to be viewed in its entirety under high magnification (x400) of a phase contrast microscope. The area of the glass slide containing the microdrop was humidified by inverting the glass slide, chamber down, on a small water bath containing PBS pre-warmed to 37°C. The hanging drop remained intact for 3-5 minutes without appreciable evaporation taking place.

When a drop containing a single trypanoson ' was selected, it was confirmed by a second observer. The glass slide was then removed from the microscope and everted. The hanging drop was enlarged by addition of more PBS from a 1 ml disposable syringe. The enlarged drop was aspirated into a 1 ml syringe containing 0.2 ml of PBS. The chamber previously containing the single trypanosome was washed twice with PBS. The initial drop together with the washings were injected intraperitoneally into a single immunosuppressed mouse. In this way about 5-10 immunosuppressed mice were injected with single trypanosomes during each cloning procedure.

Injected mice were examined for patent parasitaemia from day 6 post infection using wet mount preparation of tail blood. Heavily infected blood was obtained by cardiac puncture from an anaesthetised infected mouse and a stabilate prepared and stored in liquid nitrogen. Clones selected for study were expanded in immunosuppressed mice and a large number of such stabilates prepared and stored in aliquots of 0.5-1.0 ml in liquid nitrogen until used.

3.7. ISOLATION AND ADAPTATION OF TRYPANOSOMA EVANSI IN VITRO

Bloodstream forms of *T. evansi* were isolated from infected mouse blood and adapted to axenic growth according to the method of Baltz *et al*, (1985). A mouse was infected with approximately 2×10^5 trypanosomes i.p., from a frozen stabilate of *T. evansi* TREU 1840. After 3 days when parasitaemia was increasing the mouse was sampled for trypanosome isolation as follows: The tail of the mouse was swabbed with 70% ethyl alcohol and allowed to dry. The tail was snipped with a pair of scissors which had been cleaned with 70% alcohol. A drop of blood was expressed

from the snipped end of the tail and 5μl collected without an anticoagulant, into a sterile bijou.

A 24-well culture plate (FALCON, UK) was previously prepared by addition of 1 ml of fresh *T. evansi* medium into each of 2 wells. 1.5 ml of sterile distilled water was added into each of the outer wells to prevent evaporation. The plate was placed in the CO_2 incubator at 37°C. A 2.5 μl aliquot of sampled blood was placed at the centre of one of the wells containing 1 ml of fresh medium. 1 ml of medium was added to the rest of the blood in the bijou and mixed using a Pasteur pipette. The trypanosomes in this suspension were counted and diluted to give a final concentration of 2×10^5/ml in 2 mls, when 1 ml of the diluted culture was added into the second well. The plate was incubated at 37°C in a gassed incubator with 5% CO_2 in air.

The culture plate was examined twice daily using phase contrast microscope for the growth of trypanosomes. The medium in each well was changed individually. The supernatant was passaged into succeeding wells of the 24-well plates according to the density of trypanosomes in each well. Initially culture medium was changed twice a day by removing 0.5-1 ml of culture and replacing with equal volume of fresh medium. Depending on the density of trypanosomes in the well culture medium could be removed from the top without agitating the entire culture, or the culture suspension was mixed using a 1 ml pipette. 0.5 ml of culture supernatant removed was passaged into a new well and 1-1.5 ml of medium added initially and thereafter maintenance continued as in older wells.

Many trypanosomes died within the first 2 days of culture but growth became stable thereafter. It was possible to change cultures once daily after the first 5 days. Cultures were passaged into new wells until there were no trace of red blood cells present in the wells. Cultures in these wells were then maintained until trypanosomes were harvested. Stabilates of healthy cultures were prepared and cryopreserved.

Cultures were judged to have become established, when they showed a steady exponential growth after dilution. Trypanosomes were then harvested into sterile

universals, counted, and seeded in T-25 culture flasks. Cultures were seeded at the density of 1×10^5 trypanosomes/ ml in 6 mls of fresh medium. For the first 2 weeks after transfer to the flasks cultures were examined and fresh medium provided every 24 hours. After establishment in the flask medium was changed every 2 or 3 days.

3.8. CULTURE CONDITIONS

Trypanosomes were grown in suspension (axenic) culture either in T-25 or T-75 flasks. The medium used was Eagle's minimum essential medium (MEM,) with Earle's salts. This medium was supplemented with 10-15% heat-inactivated (56°C, 30 minutes) donor horse serum, 2 μM L-glutamine, hypoxanthine (HT), 100 μM bathocuproinedisulfonic acid, non-essential amino acids, 100 μM L-cysteine, 100 μM monothioglycerol and 0.2% glucose. Cultures were maintained routinely by diluting to approximate density of either 10^5/ml over 2 days or 10^4/ml over 3 days, in 6-12 mls (for T-25 flasks) or 75 mls (for T-75 flasks) fresh medium. Flasks were incubated at 37°C and 5% CO_2 in air.

3.9. CRYOPRESERVATION OF TRYPANOSOMA EVANSI CULTURES.

T. evansi cultures which were actively growing in the log phase, at a density of 1.5-2×10^6/ml of medium were harvested for cryopreservation. After examination of the culture in either T-25 or T-75 culture flask the culture suspension was mixed using a flamed sterile Pasteur pipette. A small volume of the culture supernatant was taken and the numbers of trypanosomes counted, in the chambers of Neubauer haemocytometer. The culture supernatant fluid from the flask was removed with glass pipette and placed into a sterile universal, and centrifuged at 1000x g for 10 minutes. The pellet was resuspended in 7.5% glycerol in fresh *T. evansi* medium to give a final density of $3-5 \times 10^6$ trypanosomes/ml. The trypanosome/glycerol mixture was dispensed into 1.8 ml cryopreservation ampoules. These were capped and placed immediately in the vapour phase of liquid nitrogen for at least 4 hours or overnight. They were then transferred into the main liquid nitrogen bank and stored at -181°C. Details of these stabilates were also kept on record cards.

3.10. INITIATION OF TRYPANOSOMA EVANSI CULTURES FROM CRYOPRESERVED STABILATES.

Frozen ampoules of stabilates were removed from liquid nitrogen and thawed as quickly as possible by placing in a pre-warmed water bath at 37°C. The culture was diluted into 8 mls of chilled (4°C) *T. evansi* culture medium in a sterile universal and centrifuged at 1000x g for 10 minutes. The pellet was resuspended in 6 mls of fresh medium. The trypanosome suspension was p' iced in a sterile 25 Cm culture flask and kept in a CO_2 incubator at 37°C and 5% CO_2 in air. The cap of the flask was kept loose overnight (or at least 2 hours) for gas to equilibrate. Fast growing cultures were changed and fresh medium provided after 24 hours. Slow growing cultures were changed after 48 hours. Flasks were changed in each case. Thereafter the cultures were maintained as routine.

3.11. CLONING OF TRYPANOSOMES IN VITRO

Trypanosoma evansi axenic bloodstream forms made resistant to Mel Cy were cloned by limit dilution. Six 96-well plates were filled with 50 µl/well of fresh *T. evansi* culture medium and placed in an incubator at 37°C and 5% CO_2 to equilibrate. *T. evansi* culture which was growing well was counted using a Neubauer haemocytometer and diluted to a density of 4 trypanosomes/ml in 20 mls of medium. The final trypanosome suspension was poured into a sterile trough and 50 µl volumes were plated out to each of the wells of 96-well plates using a multichannel Finn pipette. Plates were incubated at 37°C, 5% CO_2 for 5-7 days. Plates were thereafter examined for growth of trypanosomes in each of the wells. Healthy-growing cloned populations were transferred individually to wells of a 24-well culture plate where they were passaged and grown for 5 days. Established clones were transferred to T-25 culture flasks and maintained routinely. Stabilates were prepared from each of the clones and stored in liquid nitrogen.

3.12. DRUGS AND CHEMICALS

Melarsenoxide cysteamine (mel Cy, Cymelarsan®) was kindly supplied by Rhone-Poulenc, France; melarsoprol (mel B, Arsobal®) was provided by Specia, Rhone Poulenc; diminazene aceturate (Berenil®) was donated by Hoechst A G, Germany; quinapyramine chloride: quinapyramine sulfate 4:3 (Antrycide prosalt®) by ICI Ltd, United Kingdom; suramin (Naganol®) was obtained from Bayer A G, Germany, and cyclophosphamide monophosphate was obtained from SIGMA.

[2-^{3}H]-adenosine (740-925 GBq/mmol^{-1} or 20-25 Ci/mmol^{-1}) was purchased from Amersham International Plc., UK, and stored at +4°C. Adenine (6-aminopurine), inosine (hypoxanthine 9-D-ribofuranoside), adenosine (9-β-D-ribofuranosyladenine) and 2'deoxyadenosine were all obtained from SIGMA. Adenine, inosine and deoxyadenosine were stored at room temperature and adenosine was stored at +4°C.

Mel B was supplied in solution in propylene glycol. It was also diluted in propylene glycol for use in all experiments. Other drugs were dissolved in double distilled, de-ionised water and filter-sterilised using 0.22 μm filter (Millipore). Quinapyramine and Berenil® were prepared and used once only on the day of experiment. Suramin and mel Cy were stored frozen at -20°C as a 10 or 100 mg/ml, or 1 mg/ml solutions respectively.

For *in vitro* assays the drug solutions were prepared at 100x final concentration required in the assay. Concentrations of diminazene aceturate were calculated as 44.5% content of Berenil®.

CHAPTER FOUR

PRODUCTION AND CHARACTERISATION OF MEL CY-RESISTANT
TRYPANOSOMA EVANSI POPULATIONS *IN VIVO* AND *IN VITRO*

4.1. INTRODUCTION

It has long been recognised that prolonged use of trypanocides for the treatment of
trypanosomosis promotes the development of drug resistant trypanosomes (Fulton
and Yorke, 1941). Consequently drug resistance has been investigated using
naturally occurring (Zhang *et al.*, 1993) or laboratory produced (Osman *et al.*, 1992)
drug resistant strains. In order to investigate the genetic or biochemical basis of drug
resistance it is necessary to have populations of trypanosomes that are identical
except for their response to a particular drug. Such populations can only be obtained
under controlled experimental conditions.

It has been shown that drug resistance is more likely to develop in
immunosuppressed animals than in hosts with intact immune systems (see Doenhoff
et al., 1991; Osman *et al.* 1992).

Besides the use of animal hosts, maintenance of drug resistant trypanosomes *in vitro*
would facilitate studies on the mechanisms of drug resistance. This would make it
possible for the various parasite adaptation mechanisms to be followed in the absence
of host interference. *In vitro* methods are increasingly being considered as useful
alternatives to animal hosts. They have an added advantage for species of
trypanosomes which are not infective to laboratory animals. However, for them to be
effective it is necessary that there is correlation of the *in vitro* results with *in vivo*
findings. To determine this there is need to undertake parallel studies in both *in vivo*
and *in vitro* systems. It is also important to ascertain that drug resistant trypanosomes
produced *in vitro* would maintain their normal characteristics when transmitted to the
animal host.

The overall aim of this study is to investigate the biochemical basis of resistance to melaminophenyl arsenicals in *T. evansi*. The specific aims presented in this chapter include the following:

1. To produce drug resistant lines of *T. evansi in vivo* and *in vitro* from a drug sensitive clone using a trivalent melaminophenyl arsenical drug, mel Cy.

2. Characterise drug resistant lines with respect to their behaviour in normal host, and sensitivity to mel Cy.

3. Determine the stability of the resistance phenotype produced by mel Cy *in vivo* and *in vitro*.

4. Determine any cross resistance between mel Cy and other commonly used trypanocides.

5. Compare the effect of different induction methods on development of drug resistance.

6. Determine any correlation between the results of *in vitro* assays with *in vivo* sensitivity tests.

4.2. *METHODS AND RESULTS*

4.2.1. *In vivo* determination of sensitivity of *Trypanosoma evansi* TREU 1840 to mel Cy

A preliminary dose titration was carried out to determine the sensitivity of *T. evansi* TREU 1840 to mel Cy, and a sub-curative dose to be used for initiation of induction of resistance.

Groups of 5 mice each were injected intraperitoneally (i.p.) with 0.2 ml of a 1:100 dilution of whole blood stabilate (containing approximately 10^5 trypanosomes), in PBS. Wet blood smear preparations of tail blood from infected mice were examined daily for patent parasitaemia.

At rising parasitaemia each group of mice was treated with one of the following doses of mel Cy: 0.01, 0.05, 0.1, 0.5 and 1 mg/kg, i.p. The drug was administered in 0.1 ml/10 gm body weight. Tail blood was again examined for parasitaemia 24 hours post treatment and subsequently three times a week, for 60 days.

From this data minimum curative dose (MCD $_{100}$) i.e. the lowest dose of mel Cy at which no relapse was detected in all treated mice throughout the period of observation, was determined.

Results of this experiment are discussed in Section 4.2.4.2.1.

4.2.2. Induction of resistance to mel Cy in *Trypanosoma evansi in vivo*

Attempts were made to generate mel Cy resistant trypanosomes from TREU 1840 using both immunosuppressed and normal (immunocompetent) mice. Immunosuppression of mice was carried out using either cyclophosphamide treatment or sublethal whole body irradiation. The relapse method of Osman *et al.*, (1992) was used in induction.

4.2.2.1.   *Induction of resistance in Cyclophosphamide-treated mice*

Five mice were each injected i.p, with 300 mg/kg cyclophosphamide, 4 hours prior to inoculation with trypanosomes.   Approximately $1x10^5$ trypanosomes in 0.2 ml suspension of whole blood diluted in PBS was injected, i.p. Wet mount preparations of tail blood of infected mice were examined daily for parasitaemia.   At rising parasitaemia mice were treated with 0.1 mg/kg mel Cy, i.p. Tail blood was examined 3 times a week for parasitaemia. Relapse parasitaemia detected was allowed to attain exponential growth.   The mouse was then bled terminally under anaesthesia, in 5 IU/ml heparin. 0.2 ml of the parasitaemic blood was passaged into each of 5 new cyclophosphamide-treated mice for the next stage of treatment.

At log phase of the new infection mice were allocated to 2 or 3 treatment groups. One group was treated with the dose at which the parasites relapsed (current dose) and the other group(s) were treated with slightly higher dose(s).   Mice were examined daily for relapse parasitaemia to be passaged into a new set of immunosuppressed mice. This process was repeated until a resistant population was obtained. Intermediate stabilates were prepared at various stages of induction.

Resistant populations derived were cloned in cyclophosphamide-treated mice.

4.2.2.1.1.   *Results of induction of resistance in cyclophosphamide-treated mice*

A summary of the progression of induction in cyclophosphamide-treated mice is shown in Figure 4.1. A high degree of resistance to mel Cy (up to 30 mg/kg) was achieved over a relatively short period of time.

At the initial steps it was possible to increase doses only slightly each time, starting from a subcurative dose of 0.1 mg/kg.   However, above 2.0 mg/kg dose the trypanosomes markedly gained resistance and it became possible to significantly increase the stepwise doses and obtain relapses in relatively short periods of time. Induction of resistance from 0.1-30 mg/kg mel Cy was achieved by 12 passages, over 5 months period.

The relapse population at 30 mg/kg mel Cy was sub-passaged in 5 cyclophosphamide-treated mice and treated with 30 mg/kg mel Cy. Infection was not

cleared by this dose. Parallel treatment was carried out in 5 normal mice and relapses obtained from 3/5 treated mice. This population was expanded in cyclophosphamide-treated mice and a stabilate prepared. This resistant line was designated CR1. From CR1 5 clones were derived in cyclophosphamide-treated mice. Two clones, designated CR1.2 and CR1.3 were expanded in cyclophosphamide-treated mice and stabilates made for further characterisation.

4.2.2.1.2.   Stabilising resistance

After preliminary tests on stability of resistance to mel Cy attempts were made to stabilise the resistance produced *in vivo*.

The CR1 parasites were again selected with 40 mg/kg mel Cy (maximum tolerated dose of mel Cy in mice) in cyclophosphamide-treated mice. The relapse population obtained was designated CR2. The CR2 were sub-passaged 11 times successively in cyclophosphamide-treated mice and treated each time with 40 mg/kg mel Cy. At the end of these passages the growth rate of the parasites was not affected by drug treatment. This population was designated CR3.

The CR3 population was tested in cyclophosphamide-treated and normal mice by infection and treatment with 40 mg/kg mel Cy. Infection was not cleared in both the normal and immunosuppressed mice, and the growth rate of the parasites was not reduced. A stabilate of the relapse population in cyclophosphamide-treated mice was expanded in 5 cyclophosphamide-treated mice. Three clones derived from this stabilate were designated CR3.1, CR3.2 and CR3.3.
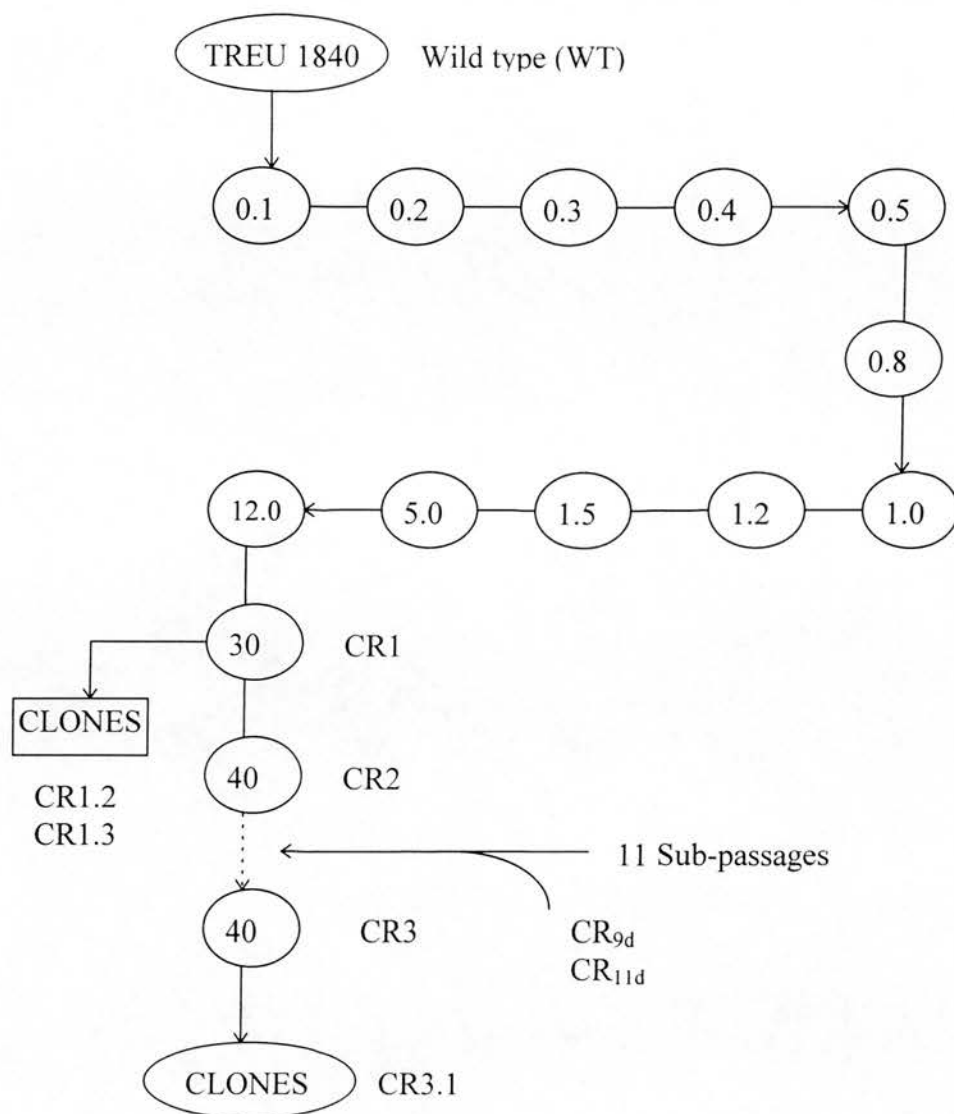
Figure 4.1 : Summary of induction of mel Cy resistance in cyclophosphamide-treated mice using subcurative dose treatment. A group of 5 mice was treated with 300 mg/kg cyclophosphamide, i.p., 4 hours prior to inoculation with trypanosomes. 0.2 ml of frozen whole blood stabilate of TREU 1840 was injected, i.p. Two to 3 days later when infections were established mice were treated with a starting subcurative dose of 0.1 mg/kg mel Cy. Wet mount preparations of tail blood of treated mice were examined daily for relapses. When a relapse was detected the mouse was bled terminally, under anaesthesia. 0.2 ml of parasitamic blood was passaged into each of a new set of 5 immunosuppressed mice. At rising parasitaemia of the new infection mice were divided into 2 or 3 treatment groups. One group was treated with the dose at which the parasites relapsed. The other group(s) was treated with slightly higher dose(s). This process was repeated until a highly resistant population was obtained.

Resistant populations at 30 and 40 mg/kg doses were cloned by injection of single trypanosomes in immunosuppressed mice.

Figures represent doses of mel Cy in mg/kg. body weight. Only doses that were continued in the induction are presented.

4.2.2.2 Induction of mel Cy resistance in immunosuppressed mice using sublethal whole body irradiation

Induction was carried out as described for the cyclophosphamide-treated mice. However mice were immunosuppressed by exposure to 600 rads gamma irradiation from a ^{137}Cs source, 24 hours prior to infection. A starting subcurative dose of 0. 1 mg/kg mel Cy was used. Smaller incremental doses of mel Cy were used. A population resistant to 30 mg/kg mel Cy (IK1, Figure 4.2.) was sub-passaged into 5 irradiated and 5 normal mice and treated with the same dose. The relapse from irradiated mice group was expanded in 5 irradiated mice and a stabilate prepared. This stabilate was cloned in cyclophosphamide-treated mice. Stabilates of clones were prepared and stored in liquid nitrogen.

4.2.2.2.1. Results of induction of resistance in immunosuppressed mice using sublethal whole body irradiation method

A summary of the procedure for induction of resistance in irradiated mice is shown in Figure 4.2. Development of resistance in this group was not as rapid at the initial stages compared to the cyclophosphamide-treated mice group. More rapid increase in doses could only be achieved above 6 mg/kg. Below this dose attempts to increase doses rapidly led to failure to obtain relapse parasitaemias.

Using this method a population selected with 30 mg/kg mel Cy was also obtained after 12 passages over a period of $5^1/2$ months. This population was designated IR1. The IR1 parasites were not cleared from circulation by treatment with 30 mg/kg mel Cy, in both normal and immunosupressed mice.

Five clones were derived from IR1. One clone, IR1.4, was expanded and characterised.
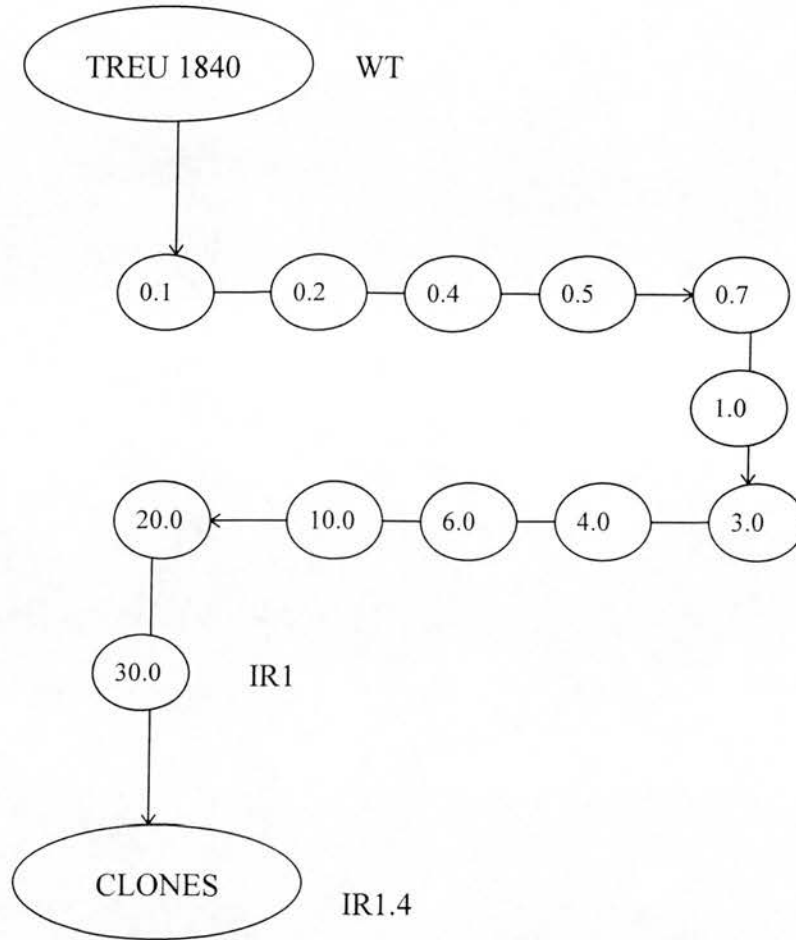
Figure 4.2 : Summary of induction of mel Cy resistance in immunosuppressed mice using sublethal whole body irradiation. A group of 5 mice was exposed to 600 rads of gamma irradiation from a ^{137}Cs source 24 hours prior to inoculation with trypanosomes. 0.2 ml of frozen whole blood stabilate of TREU 1840 was injected, i.p. Two to 3 days later when infections were established mice were treated with a starting subcurative dose of 0.1 mg/kg mel Cy, i.p. Wet mount preparations of tail blood of treated mice were examined daily for relapses. When a relapse was detected the mouse was bled terminally, under anaesthesia. 0.2 ml of parasitaemic blood was passaged into each of a new set of 5 immunosuppressed mice. At rising parasitaemia of the new infection mice were divided into 2 or 3 treatment groups. One group was treated with the dose at which the parasites relapsed. The other group(s) was treated with slightly higher dose(s). This process was repeated until a highly resistant population was obtained. A population resistant to 30 mg/kg dose was cloned by injection of single trypanosomes in immunosuppressed mice.

Figures in circles represent doses of mel Cy in mg/kg administered in 0.1 ml/10 kg body weight, i.p. Only doses that were continued in the induction are presented. Arrows indicate trend of increasing subcurative doses.

4.2.2.3. *Induction of mel Cy resistance in immunocompetent mice*

Induction procedure was similar to that described for immunosuppressed mice. However, non-immunosuppressed mice were used in attempt to produce drug resistance. Drug doses were increased very slowly. A dose level was repeated several times to ensure relapses on the next dose.

4.2.2.3.1 Results of induction of resistance in immunocompetent mice

A summary of the induction procedure in immunocompetent mice is shown in Figure 4.3. The development of resistance in this group was exceptionally slow. It was necessary to carry out 2 or more sub-passages at the same dose to obtain relapses. Relapse populations took very long time to appear and produced transient and scanty parasitaemias. Failure to obtain relapse parasitaemias at various dose levels was common.

It took $5^1/2$ months to obtain relapses up to 1.0 mg/kg mel Cy, the curative dose for the drug sensitive clone. Relapses were obtained at 1.4 mg/kg mel Cy but only after 24 months of induction. Beyond this dose it was even more difficult to obtain relapses. It became necessary to discontinue this induction process for lack of time.

4.2.3. Induction of mel Cy resistance in *Trypanosoma evansi in vitro*

T. evansi TREU 1840 bloodstream forms were isolated from infected mouse blood and adapted to axenic *in vitro* growth as described in Section 3.8. Trypanosomes were maintained in the continuous presence of increasing subinhibitory concentrations of mel Cy, starting from 0.1 ηg/ml. Fresh drug was introduced every 2 or 3 days when the culture medium was replenished. From the initial dose of 0.1 ηg/ml the concentration of mel Cy was increased gradually by doubling the concentration added to the suspension cultures. Cultures were maintained in one concentration of drug until they achieved similar growth rate as the wild type cultures maintained in the absence of drug. The resistant population (MCR1) obtained was cloned by limit dilution method. Stabilates of clones were prepared and stored in liquid nitrogen for further characterisations.
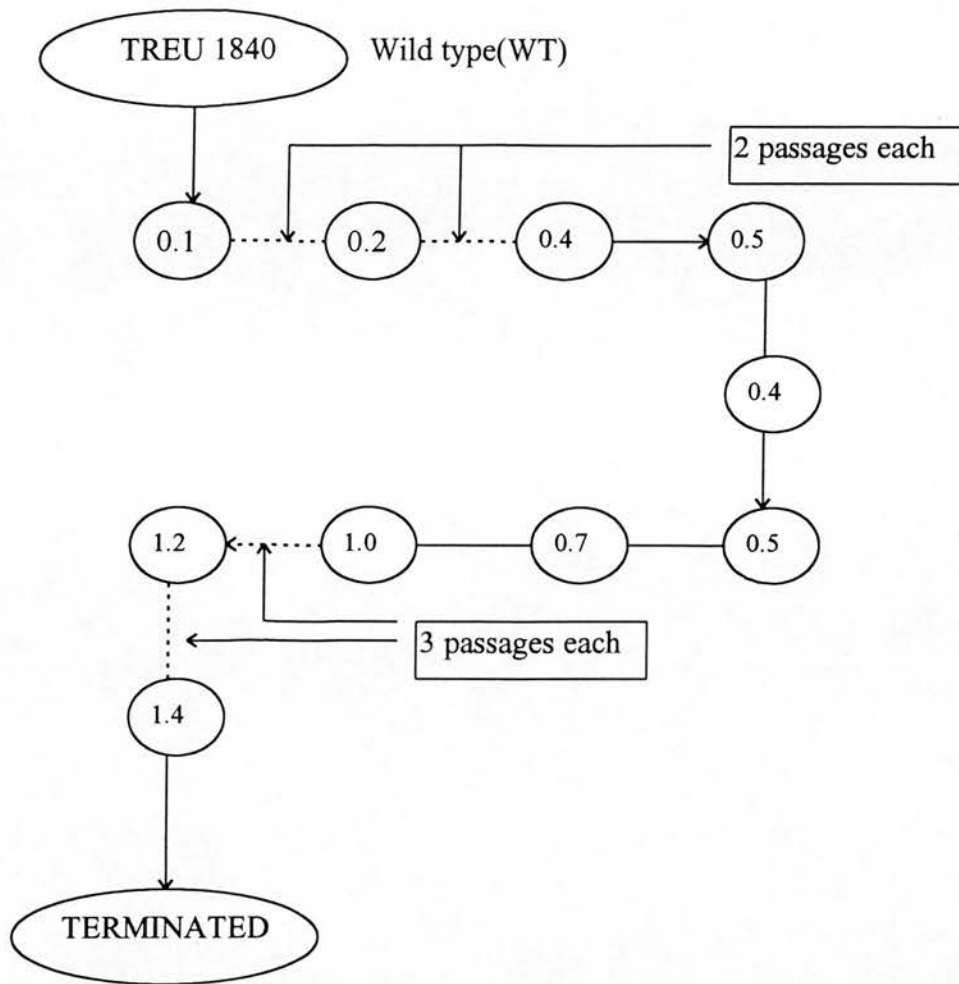
Figure 4.3: Induction of mel Cy resistance in immunocompetent mice using subcurative dose treatment and relapse method. 5 normal mice were injected with 0.2 ml of frozen whole blood stabilate of TREU 1840, i.p. Two to 3 days later when infections were established mice were treated with a starting subcurative dose of 0.1 mg/kg mel Cy, i.p. Wet mount preparations of tail blood of treated mice were examined daily for relapses. When a relapse was detected the mouse was bled terminally, under anaesthesia. 0.2 ml of parasitaemic blood was passaged into each of a new set of 5 immunosuppressed mice. At rising parasitaemia of the new infection mice were divided into 2 or 3 treatment groups. One group was treated with the dose at which the parasites relapsed. The other group(s) was treated with slightly higher dose(s). Induction was discontinued after 24 months with relapses only at 1.4 mg/kg dose.

Figures in boxes represent doses of mel Cy in mg/kg body weight administered in 0.1 ml/10 kg body weight. Only doses that were continued in the induction are presented.

4.2.3.1. Results of induction of mel Cy resistance in Trypanosoma evansi in vitro

The culture-adapted TREU 1840 (WTAX) was cultivated in the continuous presence of increasing concentrations of mel Cy starting from 0.1 ηg/ml. A population of trypanosomes that tolerated the continuous presence of 20 ηg/ml of mel Cy, designated MCR1 was obtained after 6 months of cultivation in the presence of the drug. A stabilate of this population was prepared and stored in liquid nitrogen. Several clones were derived from the MCR1 line. One clone, MCR1.1, was characterised.

A summary of the derivation of all the drug resistant lines is given in Table 4.1

Table 4.1: A summary of derivation of mel Cy resistant lines of *Trypanosoma evansi in vivo* and *in vitro*.

Unselected T. evansi	Highest selection dose of mel Cy	Resistant line	Resistant clone	Selection system
TREU 1840 (WT)	30 mg/kg	IR1	IR1.4	Irradiated mice
	30 mg/kg	CR1	CR1.2 CR1.3	Cyclophosphamide-treated mice
	40 mg/kg	CR2	-	Cyclophosphamide-treated mice
	40 mg/kg	CR3	CR3.1	Cyclophosphamide-treated mice
TREU 1840 (WTAX)	20 ηg/ml	MCR1	MCR1.1	*In vitro* culture

Legend: The unselected drug sensitive *T. evansi* TREU 1840 maintained in mice (WT) was passaged successively in either cyclophosphamide-treated mice or gamma irradiated mice. Infected mice were treated with increasing subcurative doses of mel Cy, i.p., and relapses obtained. Relapses at the highest selection dose were stabilated and cloned in immunosuppressed mice. TREU 1840 was also adapted to *in vitro* axenic growth (WTAX) and selected using increasing sub-inhibitory concentrations of mel Cy in culture. The resistant population obtained was cloned *in vitro* by limit dilution method.

4.2.4. Characterisation of mel Cy resistant trypanosome lines *in vivo*

4.2.4.1. Mice infections

The behaviour of drug resistant trypanosome lines derived using cyclophosphamide-treated mice (CR1, CR2) irradiated mice (IR1) and cultures (MCR1) was studied in comparison to the wild type (WT) parasites. A group of 5 non-immunosuppressed mice were infected with approximately 10^5 trypanosomes/mouse from each of drug resistant lines. Infected mice were examined daily for parasitaemia using wet mount preparation of tail blood, up to the time of death. (Mice were killed under approved conditions when there was evidence of distress as a result of infection.) The characteristics studied included infectivity to normal mice, prepatent period (time between injection with trypanosomes and detection of parasites in the blood), and virulence (determined by the number of days to death after infection).

4.2.4.1.1   Results of characterisation of drug resistant trypanosome lines in mice

A summary of the characteristics of the drug resistant lines in mice is shown in Table 4.2

Infectivity: The drug resistant lines derived from either methods of induction namely, using immunosuppressed mice or *in vitro* cultivation, all maintained 100% infectivity to normal mice.

Prepatent period: The average prepatent periods of the resistant populations derived from cyclophosphamide-treated mice (CR1 and CR2) and gamma-irradiated mice (IR1) ranged from 1.4-1.6 days. The mean prepatent period for the WT parasites was 1.4 days.

Virulence: In infections with CR1 average number of days to death was 12.6 (range 11-15); CR2 was 8.6 (range 5-14), IR1 was 10.6 (range 8-17) and WT was 5.4 days (range 4-7)

Table 4.2: *In vivo* characteristics of *Trypanosoma evansi* isolate and its drug resistant derivatives.

T. evansi line	Number of mice infected	Infectivity	Prepatent period (days)		Number of days to death of infected mice	
			Range	Mean	Range	Mean
WT	5	5/5	1-2	1.4	4-7	5.4
CR1	5	5/5	1-2	1.6	11-15	12.6
CR2	5	5/5	1-3	1.4	5-14	8.6
IR1	5	5/5	1-3	1.4	8-17	10.6

Legend: Five normal mice were each injected, i.p., with approximately 1×10^5 trypanosomes in 0.2 ml of diluted stabilate of each of the resistant lines. Stabilates were diluted 1:100 in PBS. Wet mount preparations of tail blood of infected mice were examined daily for parasitaemia, up to the time of death. Infectivity was determined as the number of mice that developed patent parasitaemias after injection with trypanosomes; prepatent period represents the number of days between injection of mice with trypanosomes and detection of the parasites in the blood.

4.2.4.2. *In vivo drug sensitivity tests*

The sensitivity to mel Cy of drug resistant trypanosome lines and the culture-adapted wild type was determined in non-immunosuppressed mice.

Groups of 5 mice were injected with approximately 1×10^5 trypanosomes per mouse, i.p., from a selected stabilate. Tail blood was examined daily after infection. At rising parasitaemia the groups of mice were treated with 10, 20, 30 or 40 mg/kg mel Cy (drug resistant lines); or, 0.25, 0.5 or 1 mg/kg mel Cy (culture-adapted wild type). Wet blood smear preparations of tail blood of treated mice were examined 24 hours after treatment, and thereafter 3 times a week for 60 days.

Minimum curative dose (MCD_{100}) was determined for each trypanosome stock. Resistance factor (RF) was calculated as the value of MCD_{100} (drug resistant)/MCD_{100} (Drug sensitive). The RF represents the number of times that the drug sensitivity of resistant clone decreased compared to the unselected (drug sensitive) clone. The higher the RF the higher the level of drug resistance.

4.2.4.2.1   In vivo response of Trypanosoma evansi isolate TREU 1840 to mel Cy

The results of the *in vivo* response of TREU 1840 (WT) to mel Cy is shown in Table 4.3A. A dose of 0.01 and 0.05 mg/kg produced relapses in all 5 treated mice in each group. With 0.01 mg/kg mel Cy parasites were not cleared from circulation and growth rate was not affected. With 0.05 mg/kg only 2/5 mice were cleared of parasites 24 hours after treatment. Infections however, relapsed after 8 and 15 days post treatment, respectively.

Doses of 0.1 and 0.5 mg/kg mel Cy produced relapses in 4/5 and 1/5 treated mice, respectively. The relapses occurred between 14-33 days (0.1 mg/kg) and 36 days (0.5 mg/kg). 1.0 mg/kg mel Cy produced complete cure in all treated mice and represents the drug sensitive dose. 0.1 mg/kg was selected as a suitable subcurative dose for initiation of induction of resistance.

4.2.4.2.2.   In vivo response of drug resistant Trypanosoma evansi to mel Cy

  In vivo-derived populations

The *in vivo* responses to mel Cy of the drug resistant trypanosomes produced in mice are shown in Tables 4.3B & C. The CR1 trypanosomes relapsed following treatment with 20, 30, and 40 mg/kg mel Cy doses respectively. Parasitaemias were not cleared from circulation in all 5 treated mice at each dose level. At doses of 20 and 30 mg/kg mel Cy, the growth rate of the parasites was not affected by the drug. However, at 40 mg/kg reduced parasitaemia (estimated by reduction in number of trypanosomes per microscope field at x400 magnification) was observed after 24 hours. The parasites however multiplied thereafter and high parasitaemias were re-established.

Similarly relapses were obtained with CR3.1 parasites when infected mice were treated with 20, 30 and 40 mg/kg mel Cy. In each group of mice treated with 20 and 30 mg/kg parasites were not cleared from circulation and growth was not affected. However, at 40 mg/kg mel Cy, parasitaemia was cleared in 1/5 mice. This mouse relapsed 23 days after treatment.

In vitro-derived populations

In the WTAX trypanosomes (which represent the drug sensitive control population for the cultured lines) 3 out of 5 treated mice relapsed at 0.25 mg/kg dose. The duration of appearance of relapse in the 3 mice averaged 13.3 days (range 11-18 days). No relapses were obtained at 0.5 and 1.0 mg/kg mel Cy. This gives a minimum curative dose for this stock of 0.5 mg/kg mel Cy (Table 4. 4A)

The MCR1.1 parasites were not cured at 4ᴜ mg/kg mel Cy (Table 4.4B). All 5 treated mice relapsed at each of 20, 30 and 40 mg/kg doses. Parasitaemias were not cleared from circulation in each of the treatment groups. Slightly reduced growth rate was observed at 40 mg/kg dose.

A: TREU 1840 (WT)

Drug dose (mg/kg)	Number of mice treated	Number of relapses	Number of days before relapse
0.01	5	5	1, 1, 1, 1, 1
0.05	5	5	1, 1, 1, 8, 15
0.1	5	4	14, 18, 18, 33
0.5	5	1	36
1.0*	5	0	NA

B: CR1

Drug dose (mg/kg)	Number of mice treated	Number of relapses	Number of days before relapse
20	5	5	1, 1, 1, 1, 1
30	5	5	1, 1, 1, 1, 1
40	5	5	1, 1, 1, 1, 1

C: CR3.1

Drug dose (mg/kg)	Number of mice treated	Number of relapses	Number of days before relapse
20	5	5	1, 1, 1, 1, 1
30	5	5	1, 1, 1, 1, 1
40	5	5	1, 1, 1, 1, 23

D: CR3.1st

Drug dose (mg/kg)	Number of mice treated	Number of relapses	Number of days before relapse
20	5	1	18
30*	5	0	NA
40	5	0	NA

Tables 4.3: *In vivo responses of Trypanosoma evansi TREU 1840 and its in vivo*-derived drug resistant derivatives, to mel Cy. Groups of 5 normal mice were infected, i.p., with 1×10^5 trypanosomes from either the unselected drug sensitive line maintained in mice (WT), panel A; the *in vivo*-derived drug resistant lines, CR1, panel B, CR3.1, panel C; or the *in vivo*-derived resistant line passaged 15 times in mice with no drug treatment, CR3.1st, panel D. At rising parasitaemia the groups of mice were treated with varying doses of mel Cy, i.p. Tail blood of treated mice was examined after 24 hours and thereafter 3 times a week, using wet mount preparations, for 60 days, with respect to appearance of patent parasitaemia .

* = Minimum curative dose (MCD_{100}); NA = not applicable;
1 = infection not cleared from circulation after treatment.

A: WTAX

Drug dose (mg/kg)	Number of mice treated	Number of relapses	Number of days before relapse
0.25	5	3	11, 11, 18
0.50	5	0	NA
1.00	5	0	NA

B: MCR1.1

Drug dose (mg/kg)	Number of mice treated	Number of relapses	Number of days before relapse
20	5	5	1, 1, 1, 1, 1
30	5	5	1, 1, 1, 1, 1
40	5	5	1, 1, 1, 1, 1

C: MCR1.1st

Drug dose (mg/kg)	Number of mice treated	Number of relapses	Number of days before relapse
10	5	5	1, 1, 1, 1, 1
20	5	5	1, 1, 1, 1, 1
40	5	2*	1, 1

Table 4.4: *In vivo* responses of the culture-adapted TREU 1840 (WTAX) and its *in vitro*-derived drug resistant derivatives, to mel Cy. Groups of 5 normal mice were infected, i.p., with 1×10^5 trypanosomes from either the unselected drug sensitive line cultivated *in vitro* (WTAX), panel A; the *in vitro*-derived drug resistant line, MCR1.1, panel B; or the *in vitro*-derived resistant line cultivated for 5 months after withdrawal of drug, MCR1.1st, panel C. At rising parasitaemia each group of mice was treated with varying doses of mel Cy, i.p. Tail blood of treated mice was examined after 24 hours and thereafter 3 times a week, using wet mount preparations, for 60 days, with respect to appearance of patent parasitaemia .

* = 3 mice died of drug toxicity.

NA = not applicable

1 = infection not cleared from circulation after treatment

4.2.5. Characterisation of drug resistant trypanosomes *in vitro*

4.2.5.1. In vitro drug assays

The sensitivity to mel Cy of both drug sensitive and drug resistant populations was determined *in vitro*, using a cell proliferation assay (PROMEGA). This assay measures growth inhibition of trypanosomes at varying concentrations of trypanocide. The principle involves the conversion of a substrate in the assay by viable cells to an easily detectable product. Specifically the assay measures the conversion of a tetrazolium substrate to a blue formazan product. The colour change is quantified using an ELISA plate Reader.

Trypanosomes in logarithmic phase of growth were either separated from infected mouse blood or harvested from culture suspensions. Cultures were diluted 24 hours prior to assay. In case of mice isolations the trypanosomes were washed by centrifugation at 1000x g for 10 minutes. The pellet was resuspended in 10 mls of *T. evansi* medium and centrifuged again for 10 minutes at 1000x g. The final pellet was resuspended in 5 mls of *T. evansi* medium for counting. Trypanosomes were counted using the Neubauer haemocytometer and the density adjusted to 2×10^5/ml using *T. evansi* medium.

The trypanosome suspension was distributed in 1 ml aliquots into 6 sterile bijoux. Six 10-fold serial dilutions of mel Cy were prepared at 100x final concentration in assay in sterile double distilled deionised water. The final concentrations of mel Cy in assays were 0.01, 0.1, 1, 10, 100, and 1000 ηg/ml.

Ten μl of each drug dilution was added to 1 ml of trypanosome suspension in a bijou. An equivalent volume of drug solvent was added to each of control cultures. 96-well tissue culture plates were prepared by adding about 150 μl of sterile distilled water in each of the outer wells. The test wells received 100 μl of the trypanosome/drug mixture. Trypanosomes in each drug concentration were incubated in triplicate while the control cultures without drug were incubated in 6 wells. Blanks consisting of *T. evansi* medium and no trypanosomes were also incubated in 6 wells. Plates were incubated at 37°C in 5% CO_2 atmosphere, for 28 hours.

At the end of incubation Promega Cell Titre Kit was used to measure the extent of cell proliferation as follows: 15 µl of the Promega Dye Solution was added to each of test wells and the plates were incubated for 4 hours at 37°C and 5% CO_2 in air. 100 µl of Promega Solubilisation Solution was added to each test well and plates were kept in humid chamber overnight (or at least 4 hours). Absorbance was read in a spectrophotometer (ELISA plate Reader) at 570 ηm.

Absorbance values of the blanks were subtracted from test wells and means of readings were calculated. Growth of trypanosomes in test wells was calculated as a percentage of growth in control wells without drug. From these data growth inhibition curves were plotted. Minimum inhibitory concentrations (IC_{50}) were determined from inhibitory curves using the GRAFIT computer software. IC_{50} represents the concentration of drug which inhibited the growth of trypanosomes by 50% compared to the control incubations with no added drug.

Resistance Factors were calculated as IC_{50} (drug resistant line)/IC_{50} (drug sensitive line). The Resistance Factor indicates the number of times the drug sensitivity of the resistant line decreased compared to the unselected clone.

4.2.5.1.1   *In vitro response of Trypanosoma evansi isolate and its drug resistant derivatives to mel Cy*

The growth inhibition curves of the drug sensitive (WT, WTAX) and their respective drug resistant derivatives CR3, MCR1.1) in the presence of increasing concentrations of mel Cy are shown in Figure 4.4. A summary of the IC_{50} values and resistance factors determined from similar curves for these and other resistant lines is shown in Table 4.5. Large increases (over 300-fold) in the level of mel Cy resistance in the drug resistant lines were observed.

In the resistant lines derived from immunosuppressed mice, mel Cy resistance increased from IC_{50} of 2.9 ηM in WT to 646.0 ηM in CR3 representing a 221-fold
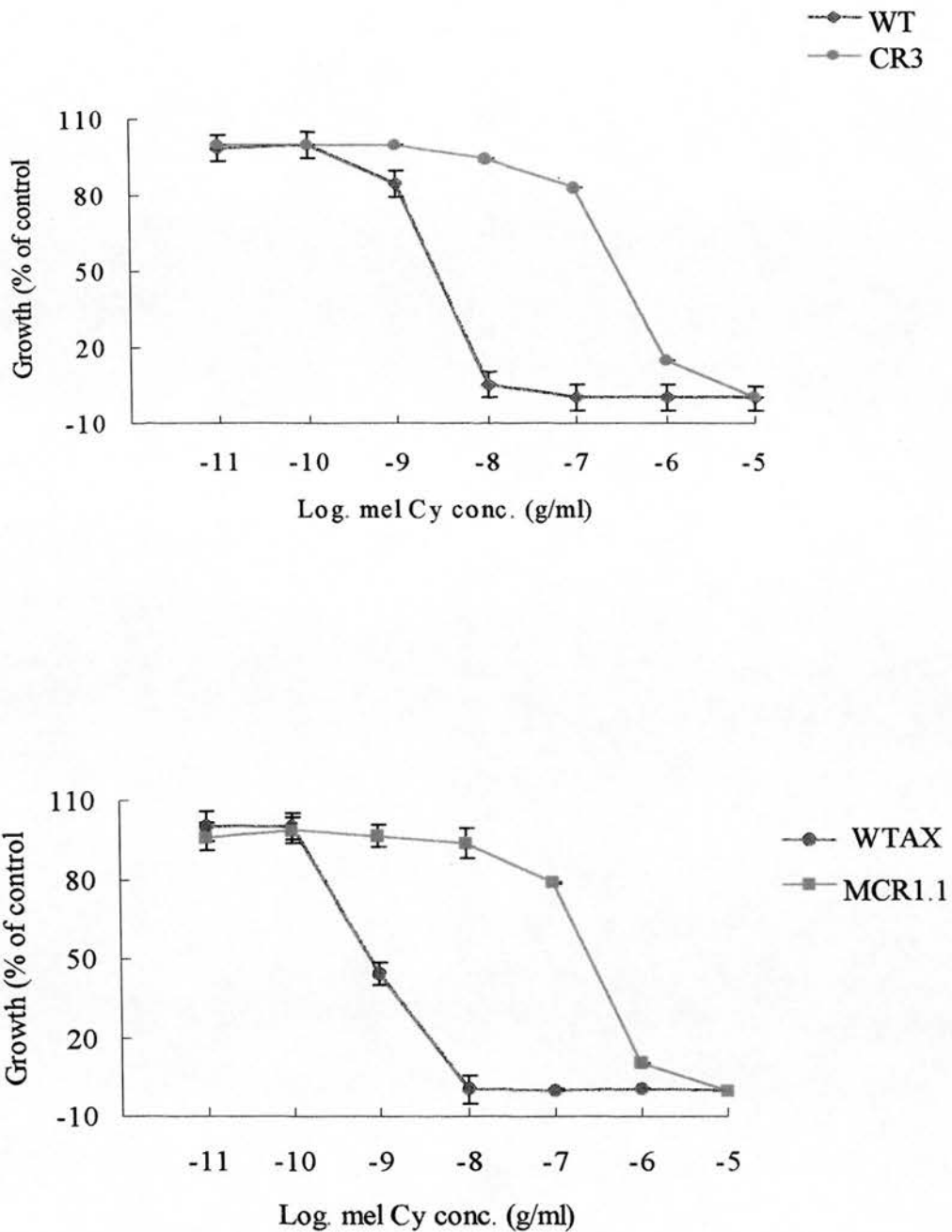
Figure 4.4: *In vitro* growth inhibition curves showing decrease in sensitivity of *in vivo*-derived (CR3, panel a) and *in vitro*-derived (MCR1.1, panel b) drug resistant *Trypanosoma evansi* to mel Cy. Trypanosomes were separated from mouse blood or harvested from cultures in exponential phase of growth. 2×10^5 trypanosomes were incubated in 100 µl of *T. evansi* culture medium at 37°C, for 28 hours, in the presence of increasing concentrations (0.01-1000 ηg/ml) of drug. The extent of proliferation of cells was determined using PROMEGA cell proliferation assay Kit. Growth at each drug concentration was calculated as percentage of control cultures with no drug added. Triplicate wells were used for each drug concentration and control cultures were incubated in 6 six wells. Bars represent standard errors of at least 3 experiments.

increase in resistance. Increases in resistance were, not uniform in all resistant lines. Resistance factors ranged from 82.0 in IR1 to 221.4 in CR3.

The three cloned populations CR1.2, CR1.3 and IR1.4 exhibited relatively higher levels of resistance compared to their respective uncloned resistant parents (CR1 or IR1). The CR3.1 clone however, showed lower RF (93.4) compared to its uncloned (CR3) parent (RF = 221.4).

Among the *in vivo*-derived resistant lines, the highest increase in resistance (RF=221.4) was observed in CR3 which had been passaged 11 times in mice in the presence of 40 mg/kg mel Cy. In the culture-derived line an IC_{50} value of 530.0 ηM in MCR1.1 compared to 1.7ηM in WTAX represented a 315-fold increase in resistance to mel Cy; being the highest increase in resistance of all the selected lines.

The sensitivities of the mouse-maintained wild type (WT) and its culture-adapted variety (WTAX) were similar. IC_{50} values were 2.9 ηM and 1.7 ηM, respectively.

Variable increases in resistance levels were attained in the drug resistant lines regardless of whether they were selected in cyclophosphamide-treated mice, irradiated mice or by *in vitro* cultivation.

Table 4.5: *In vitro* response of *Trypanosoma evansi* isolate and its drug resistant derivatives to mel Cy.

T. evansi unselected line	Mel Cy-resistant line	IC$_{50}$ (ηM)	Resistant factor (RF)
TREU 1840 WT		2.9	
	CR1	260.0	89.0
	CR1.2	572.0	195.9
	CR1.3	393.3	135.6
	CR3	646.0	221.4
	CR3.1	270.6	93.4
	IR1	240.0	82.2
	IR1.4	486.4	167.7
WTAX		1.7	
	MCR1.1	530.0	315.0

Legend: Trypanosome cultures in log phase of growth were diluted to a density of 2×10^5/ml in *T. evansi* culture medium. Cultures were incubated in 100 μl volumes in 96-well culture plates in the presence of varying concentrations of mel Cy, at 37°C and 5% CO_2 in air, for 28 hours. Control incubations with no added drug were included in each experiment. Test cultures were incubated in triplicate and control cultures in 6 wells. The extent of proliferation of cells at each drug concentration was measured using a cell proliferation assay kit (POMEGA). Percentage growth was calculated and growth inhibition curves plotted. IC$_{50}$ values were determined using the GRAFIT computer software. Data values represent average of at least 2 experiments.

IC$_{50}$ = concentration of drug which inhibited growth of trypanosomes by 50% compared to control growth in absence of drug.

RF = resistance index calculated as value of the IC$_{50}$ (drug resistant line)/IC$_{50}$ (drug sensitive line). It represents the number of times the drug sensitivity of the resistant line has decreased compared to the unselected line.

4.2.6. Stability of resistance studies

4.2.6.1. *Stability of resistance after transmission to immunocompetent mice*

The drug resistant lines derived in immunosuppressed mice (CR1, CR3, CR3.1, IR1) and in culture (MCR1.1) were each passaged into immunocompetent mice. 1×10^5

trypanosomes from either the whole blood or culture stabilate were injected into a group of 5 normal mice, i.p. Mice were examined for patent parasitaemia using wet blood smear preparations of tail blood. At rising parasitaemia each group of mice was treated with 30 or 40 mg/kg mel Cy. The treated mice were examined for 60 days for any relapse parasitaemias.

4.2.6.2. *Stability after several passages in the absence of drug pressure*

The CR3.1 parasites were passaged 15 times, over a period of 12 weeks, in normal mice with no drug treatment.

Whole blood stabilate of CR3.1 parasites was diluted 1:100 in PBS and 0.2 ml of diluted stabilate injected into each of 2 mice. The mice were examined for development of parasitaemia. At high parasitaemia (usually 5-7 days post infection) a mouse was bled under general anaesthesia. Parasitised blood obtained was diluted 1:100 in PBS and 0.2 ml injected into each of a new set of two mice. This process was repeated until a population that had been passaged through 15 sets of mice with no drug treatment (CR3.1st) was obtained. Intermediate stabilates were prepared at the 5th and 10th passages.

The culture-derived clone MCR1.1 was passaged by continuous cultivation in *T. evansi* medium for 5 months with no addition of drug in the culture medium. This population was designated MCR1.1st.

The drug sensitivities of the two passaged lines were tested in mice by infection and treatment with 10, 20 30, and 40 mg/kg mel Cy; and *in vitro* using the *in vitro* drug sensitivity assay.

In addition the MCR1.1st. line was tested for stability by re-introduction of the selective dose of mel Cy (20 ηg/ml) into the culture medium, after cultivation for 5 months in absence of drug.

4.2.6.3   Results of studies on stability of mel Cy-induced resistance

4.2.6.3.1.   Stability upon transmission to immunocompetent hosts.

Infections with both CR1 and IR1 resistant lines were not cured by their selection dose of 30 mg/kg mel Cy. In CR1 relapses occurred in 3/5 treated mice. In IR1 parasites were not cleared from circulation in 5/5 treated mice. Similarly, all 5 treated mice were not cured when CR3 and CR3.1 lines were each passaged to 5 immunocompetent mice and treated with 40 mg/kg mel Cy.

The *in vitro*-derived MCR1.1 was also not cured by 40 mg/kg mel Cy in normal mice. Parasitaemias were not cleared from circulation in all the 5 treated mice. However a slight reduction in levels of parasitaemia was observed.

4.2.6.3.2.   Instability in vivo of the in vivo-derived mel Cy resistance after several passages through mice or cultivation in the absence of drug pressure.

The drug sensitivities of the resistant lines passaged 15 times through normal mice (CR3.1st) or cultivated *in vitro* for 5 months (MCR1.1st), in the absence of drug pressure were tested for resistance in normal mice. A reduction in resistance to mel Cy was observed in the *in vivo*-derived drug resistant clone (Table 4.3D). Whereas the unpassaged clone CR3.1 was not cured by 40 mg/kg mel Cy complete cure of CR3.1st was obtained with 30 mg/kg dose and only 1/5 treated mice relapsed at 20 mg/kg dose. The single relapse occurred 18 days post treatment. In the unpassaged resistant line parasites were not cleared in all 5 treated mice at 20 mg/kg mel Cy.

4.2.6.3.3   Stability in vivo of the in vitro-derived mel Cy resistance after several passages through mice or cultivation in the absence of drug pressure.

No evidence of loss or reduction of resistance *in vivo* was observed in the culture-derived resistant clone (MCR1.1st) after cultivation for 5 months in the absence of drug (Table 4.4C). These parasites were not cured by 40 mg/kg mel Cy, similar to the unpassaged clone (MCR1.1). Parasites were not cleared from circulation following treatment and growth rate was not affected.

4.2.6.3.4 Instability in vitro of the in vivo-derived mel Cy resistance after several passages through mice or cultivation in the absence of drug pressure

A marked reduction in IC_{50} was observed in the *in vivo*-derived resistant line (CR3.1st) after 15 passages in mice in absence of drug treatment (Table 4.6). A mean IC_{50} of 8.3 ηM was obtained in comparison to 270.6 ηM in the unpassaged (CR3.1) line. This represents a 32.6-fold reduction in tolerance of the drug compared to the unpassaged line.

Table 4.6: Stability of mel Cy-induced resistance in *Trypanosoma evansi* after several passages in absence of drug pressure.

T. evansi unselected line	Mel Cy resistant derivatives	IC_{50} (ηM)	Resistant factor (RF)
WT		2.9	
	CR3.1st.	8.3	2.9
WTAX		1.7	
	MCR1.1 st.	345.0	202.9

Legend: Trypanosome cultures in log phase of growth were diluted to a density of 2×10^5/ml in *T. evansi* culture medium. Cultures were incubated in 100 µl volumes in 96-well culture plates in the presence of varying concentrations of mel Cy, at 37°C and 5% CO_2 in air, for 28 hours. Control incubations with no added drug were included in each experiment. Test cultures were incubated in triplicate and control cultures in 6 wells. The extent of proliferation of cells at each drug concentration was measured using a cell proliferation assay kit (POMEGA). Percentage growth was calculated and growth inhibition curves plotted. IC_{50} values were determined using the GRAFIT computer software. Data values represent average of at least 2 experiments.

IC_{50} = concentration of drug which inhibited growth of trypanosomes by 50% compared to control growth in absence of drug.

RF = resistance index calculated as value of the IC_{50} (drug resistant line) / IC_{50} (drug sensitive line). It represents the number of times the drug sensitivity of the resistant line has decreased compared to the unselected line.

4.2.6.3.5 Stability in vitro of in vitro-derived mel Cy resistance after several passages through mice or cultivation in the absence of drug pressure

The *in vitro* response of the culture-derived resistant clone after cultivation for 5 months in the absence of drug pressure (MCRcl.1st) is also shown in Table 4.6. The IC_{50} of 345.0 ηM was only 1.5-fold less than that of the unpassaged line (530.0 ηM). This shows that resistance in this population was stable within this period.

Following re-introduction of the selective dose (20 ηg/ml mel Cy) after 5 months of withdrawal from cultures the MCR1.1st parasites continued to grow at this concentration for 2 weeks. Reduced growth of trypanosomes was observed in the first few days after re-introduction of drug. Thereafter there was recovery and cultures attained similar growth rate as the control cultures with no added drug.

4.2.7. Cross- resistance between mel Cy and other trypanocides

The *in vitro* sensitivities of mel Cy resistant *T. evansi* lines to other commonly used trypanocides were determined in comparison to those of the unselected lines. The trypanocides tested included mel B, diminazene aceturate (Berenil®), quinapyramine and suramin. Details of their sources are given in Section 3.3

4.2.7.1.   *In vitro drug assays*

In vitro drug sensitivity assays to assess for cross-resistance were carried out as described in Section 4.2.5.1. However, several drugs were used apart from mel Cy. Drug dilutions were prepared as described below.

Quinapyramine was prepared from a 1 mg/ml stock solution for use at the following final concentrations in assay. 0.01, 0.1, 1,10, 100, 1000 ηg/ml.

Berenil® was prepared as a 1 mg/ml solution. This was used as the highest dilution for the assay. Final Berenil concentrations in the assay included 0.1, 1, 10, 100, 1000 and 10,000 ηg/ml.

Suramin was used at the same final concentrations as Berenil®. Dilutions were made from either a 10 mg/ml or 100 mg/ml frozen stock solutions.

Mel B dilutions were prepared in propylene glycol beginning with a 1 mg/ml dilution. Final concentrations in the assay were the same as in Berenil® and suramin.

4.2.7.2 Results of cross resistance between mel Cy and other trypanocides in vitro

Growth inhibition curves of mel Cy sensitive and mel Cy resistant trypanosomes incubated with increasing concentrations o. mel B, Berenil, quinapyramine and suramin, are shown in Figures 4.5 a-d. The mean IC_{50} values determined from similar curves are given in Table 4.7. A summary of IC_{50} showing cross resistance between mel Cy and other trypanocides in two resistant lines (CR3 and MCR1.1) is shown in Figures 4.6 and 4.7, respectively.

4.2.7.2.1 Cross-resistance between mel Cy and mel B (Figure 4.5a)

Moderate to high levels of cross-resistance to mel B were expressed *in vitro* in the mel Cy resistant populations. The lowest increase (11-fold) in resistance to mel B was observed in CR1 parasites which showed 89-fold increase in resistance to mel Cy (Table 4.7). The highest increase (48-fold) in resistance to mel B was found in the IR1 population which acquired 82-fold increased resistance to mel Cy. Intermediate increases of 14-fold and 36-fold resistance to mel B were observed in CR1.2 and CR3, respectively. No evidence of increased resistance to mel B was observed in the MCR1.1 parasites.
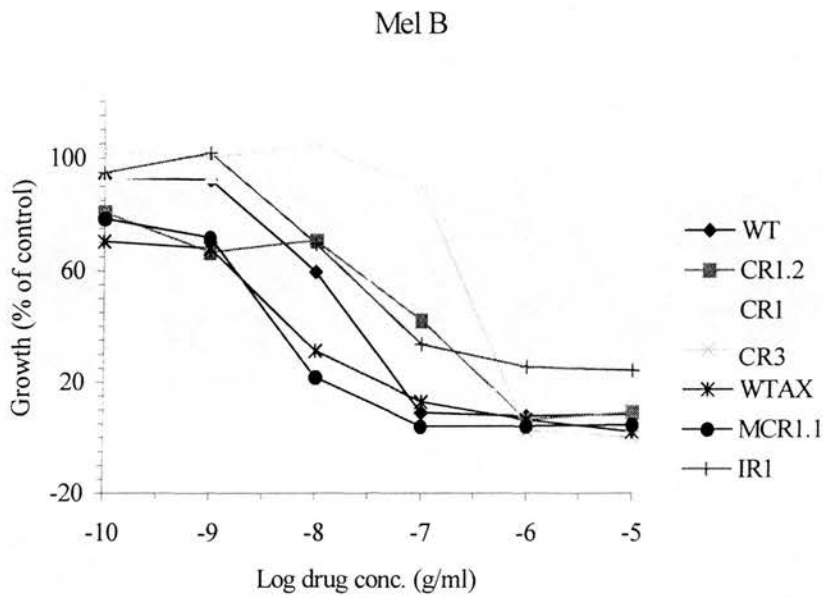
Mel B

Figure 4.5a: *In vitro* growth inhibition curves of mel Cy sensitive and mel Cy resistant *Trypanosoma evansi* at different concentrations of Mel B.

4.2.7.2.2   Cross-resistance between mel Cy and diminazene aceturate (Berenil®, Figure 4.5b)

In vivo derived resistant lines:

High levels of cross resistance to Berenil® were detected in all the drug resistant populations tested. An increase in mel Cy resistance of 89-fold in CR1 line was accompanied by 30-fold increase in resistance to Berenil®. Increase of 196-fold resistance to mel Cy in CR1.2 was also accompanied by 24-fold increases in resistance to Berenil®. The increased resistance to mel Cy in CR1.2 (RF=195.9) compared to uncloned parent (CR1, RF= 89.0) was not reflected in higher resistance to Berenil® in the same stock. However in CR3 an increase in mel Cy resistance of 221-fold was accompanied by a high (53-fold) increase in resistance to Berenil®. An 82-fold increase in resistance to mel Cy in IR1 was also accompanied by 57-fold increase in resistance to Berenil®.

Berenil

Figure caption (right of graph):

Figure 4.5b: *In vitro* growth inhibition curves of mel Cy sensitive and mel Cy resistant *Trypanosoma evansi* incubated with different concentrations of Berenil.

Legend: WT, CR1.2, CR1, CR3, WTAX, MCR1.1, IR1

In vitro derived resistant lines:

In the *in vitro*-derived drug resistant line (MCR1.1) relatively low level of cross-resistance to Berenil® was detected. A 315-fold increase in resistance to mel Cy in this line was accompanied by a 25-fold increase in resistance to Berenil®.

4.2.7.2.3   *Cross-resistance between mel Cy and quinapyramine (Figure 4.5c)*

Low cross-resistance to quinapyramine in the *in vivo*-derived drug resistant trypanosomes:

A low increase in resistance to quinapyramine was observed in the drug resistant lines derived *in vivo*, ranging from 2.6-fold in CR1 to 5.9-fold in CR1.2 parasites.

Lack of cross-resistance between mel Cy and quinapyramine in the *in vitro*-derived resistant lines:

No increase in resistance to quinapyramine was observed in the MCR1.1 resistant line. The IC_{50} (1.6 ηM) was the same (1.6 ηM) as the drug sensitive (WTAX) stock.
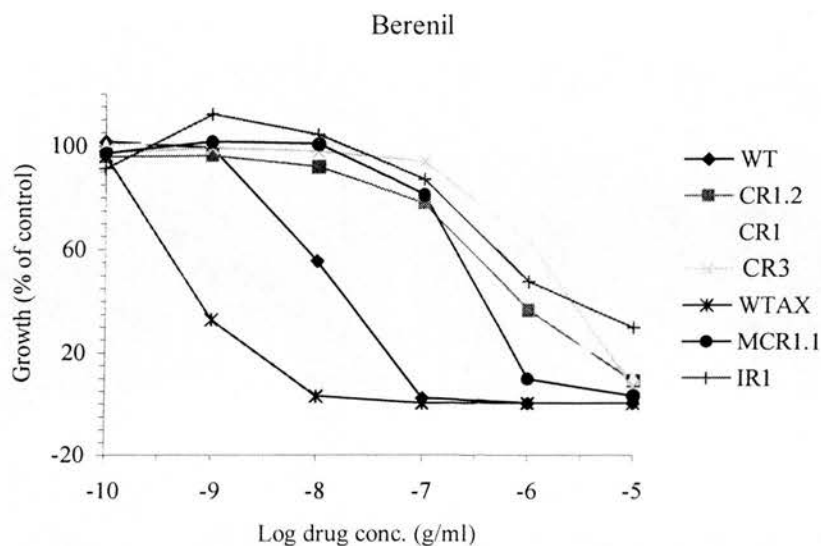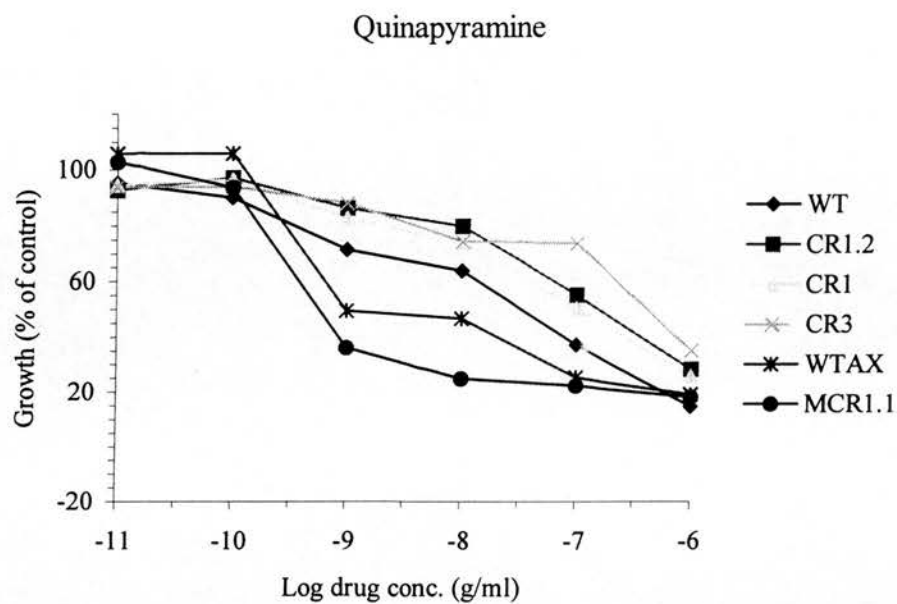
Quinapyramine

Figure
4.5c: *In vitro* growth inhibition curves of mel Cy sensitive and mel Cy resistant *Trypanosoma evansi* incubated with different concentrations of Quinapyramine.

4.2.7.2.4   Lack of cross-resistance between mel Cy and suramin in vitro (Figure 4.5d)

No evidence of increased resistance to suramin was found in the mel Cy resistant lines derived *in vivo*. Although a 2-fold reduction in sensitivity to suramin was observed in CR3 this falls within the normal limits of variability of the assay.
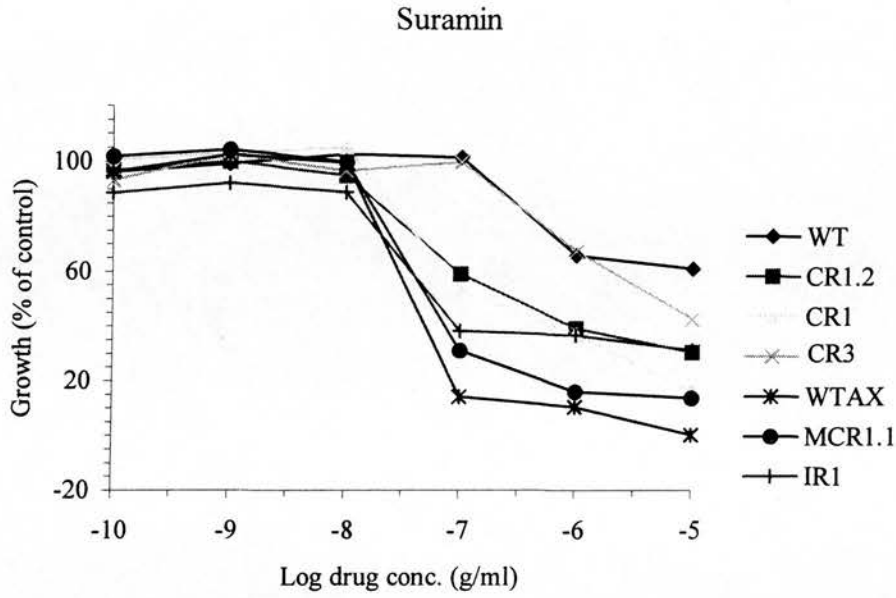
Suramin

Figure
4.5d: *In vitro* growth inhibition curves of mel Cy sensitive and mel Cy resistant *Trypanosoma evansi* incubated with different concentrations of Suramin

On the contrary two of the *in vivo*-derived resistant lines showed moderate to marked increase in sensitivity to suramin ranging from 2.3-fold increase in CR1 line to 53-fold increase in IR1 line.

There was no change in sensitivity to suramin in the *in vitro*-derived resistant line (MCR1.1, IC_{50} = 41.3 ηM) compared to its unselected clone (IC_{50}= 38.5 ηM).
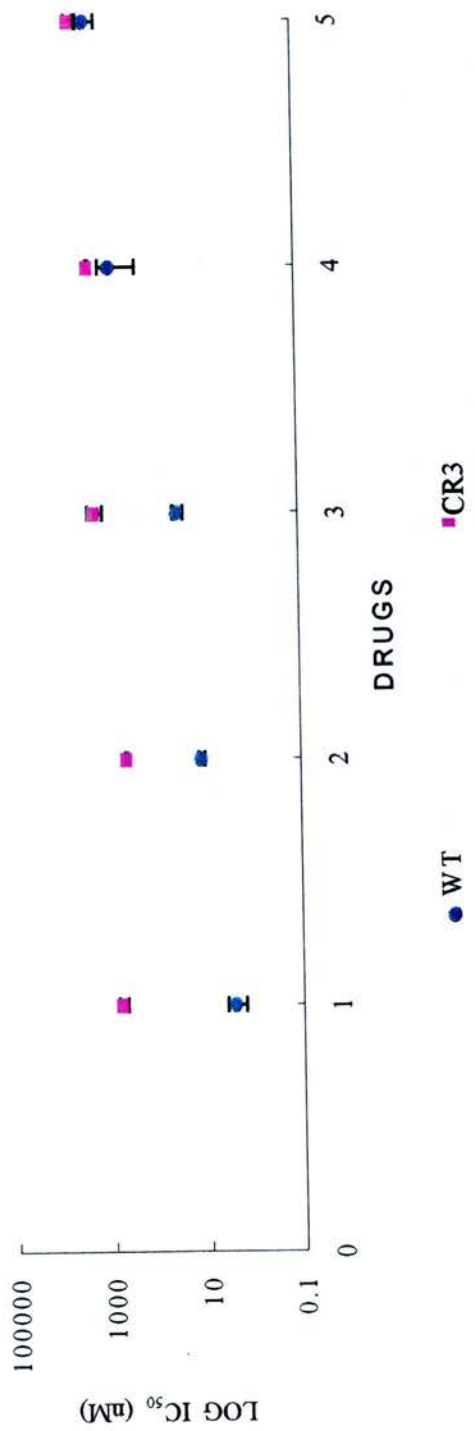
Figure 4.6: Cross resistance between mel Cy and other trypanocides. Trypanosomes separated from mice blood were incubated at a density of 2×10^5/ml of medium in the presence of varying concentrations of each drug, at 37°C and 5% CO_2 in air, for 28 hours. Each drug concentration was incubated in triplicate. Control cultures with no drug added were incubated in 6 wells. Growth was measured using cell proliferation assay (PROMEGA). Growth at each drug concentration was calculated as percentage of growth of trypanosomes in the absence of drug. IC_{50} values were determined from growth inhibition curves using the Grafit computer software. Each data point represents mean IC_{50} from at least 3 experiments.

1 = mel Cy, 2 = mel B, 3 = Berenil®, 4 = quinapyramine and, 5 = suramin.
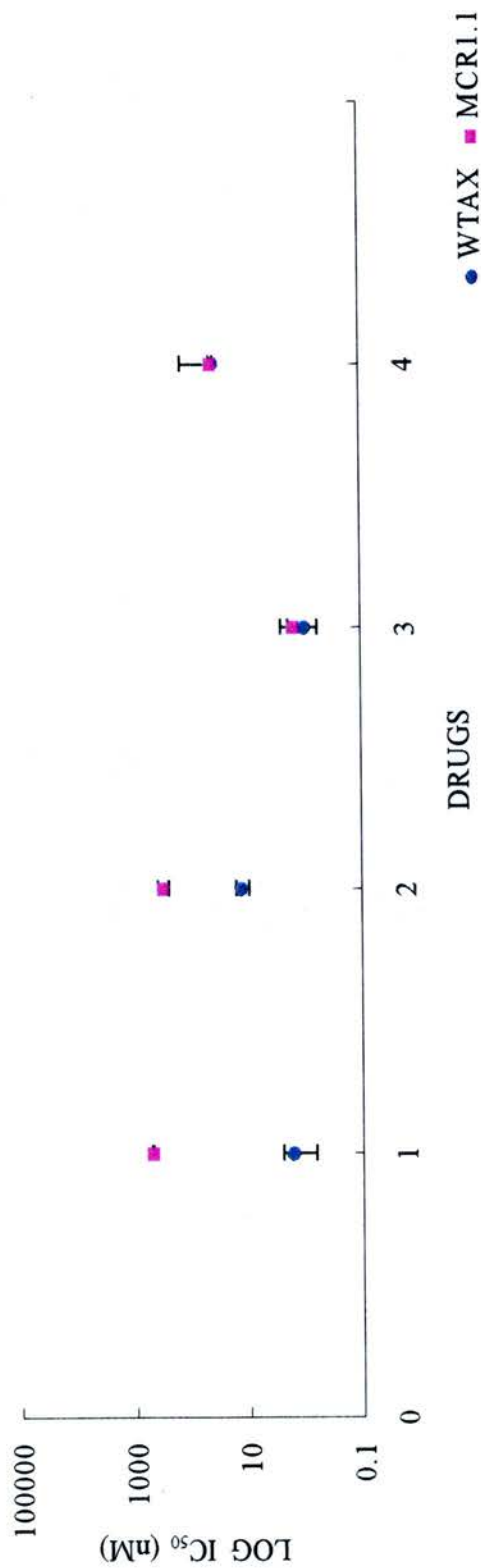
Figure 4.7 : Cross resistance between mel Cy and other trypanocides. Culture-adapted trypanosomes were incubated at density of 2×10^5/ml of medium in the presence of varying concentrations of each drug, at 37°C and 5% CO_2 in air, for 28 hours. Each drug concentration was incubated in triplicate. Control cultures with no drug added were incubated in 6 wells. Growth was measured using cell proliferation assay (PROMEGA). Growth at each drug concentration was calculated as percentage of growth of trypanosomes in the absence of drug. IC_{50} values were determined from growth inhibition curves using the Grafit computer software. Each data point represents mean IC_{50} from at least 3 experiments.

1 = mel Cy, 2 = Berenil®, 3 = quinapyramine and, 4 = suramin.

Table 4.7: Cross-resistance between mel Cy and other trypanocides.

T. evansi lines	IC_{50} (ηM)				
	Mel Cy	Mel B	Berenil	Quinapyramine	Suramin
WT	2.9	13.0	35.7	768.0	2240.0
CR1	260.0	148.0	1059.0	2023.0	980.0
CR1.2	572.0	175.0	889.0	4560.0	2800.0
CR3	646.0	468.0	1904.0	2215.0	4550.0
IR1	240.0	493.0	2040.0	-	42.0
WTAX	1.7	7.0	13.6	1.6	38.5
MCR1.1	530.0	-	340.0	1.6	41.3

Legend: Trypanosome cultures in exponential phase of growth were diluted to a density of 2×10^5/ml in *T. evansi* culture medium. Cultures were incubated in 100 µl volumes in 96-well culture plates in the presence of varying concentrations of each trypanocide, at 37°C and 5% CO_2 in air, for 28 hours. Control incubations with no added drug were included in each experiment. Test cultures were incubated in triplicate and control cultures in 6 wells. The extent of proliferation of cells at each drug concentration was measured using a cell proliferation assay kit (POMEGA). Percentage growth was calculated and growth inhibition curves plotted. IC_{50} values were determined using the GRAFIT computer software. Data values represent average of at least 2 experiments.

IC_{50} = Concentration of drug that inhibited growth of trypanosomes by 50% compared to control growth in absence of drug.

4.2.8. Test for occurrence of naturally resistant sub-populations of *Trypanosoma evansi* TREU 1840

To determine if sub-populations resistant to mel Cy were originally present in *T. evansi* TREU 1840 3 attempts were made to select the culture-adapted wild type using 20 ηg/ml concentration of mel Cy. Drug was introduced into cultures once when the medium was changed. Cultures were thereafter carefully maintained and examined for surviving trypanosomes.

Parasites that tolerated 20 ηg/ml concentration of mel Cy were not obtained from the original isolate.

4.2.9   Modification of sensitivity to trypanocides in culture-adapted *Trypanosoma evansi*

A general increase in drug sensitivity was observed in the culture-adapted *T. evansi* (WTAX) when compared to the mouse-maintained wild type (WT, Table 4.7). Low increases of 1.7-fold, 1.9-fold and 2.6-fold resistance to mel Cy, mel B and Berenil®, respectively were observed. However, marked increases in sensitivity to quinapyramine (up to 480-fold) and suramin (up to 58-fold) were also observed.

4.4. DISCUSSION

Induction of high levels of mel Cy resistance in *T. evansi* was achieved using subcurative dose treatment and relapse method in immunosuppressed mice. Similar levels were attained by *in vitro* cultivation of trypanosomes in the presence of increasing sub-inhibitory concentrations of drug. A 40-fold increase in resistance *in vivo* and over 300-fold increase *in vitro* were observed.

Induction of resistance experimentally in this study did not cause major changes in the natural characteristics of the parasites, apart from modifying their drug sensitivity. This makes them suitable material for studies of mechanisms of drug resistance. It also indicates that the method by which the parasites acquired resistance experimentally is a true reflection of their mechanisms of acquisition of resistance in nature. Hence valid conclusions can be made from observations on these parasites.

The decreased virulence observed in the resistant trypanosomes may be a result of decreased growth rate. Attainment of high parasitaemias was observed to be delayed in the resistant compared to the unselected line. Reduced growth rate in *T. evansi* made resistant to suramin was reported by Mutugi *et al.*, (1996). The growth rate of mel Cy resistant trypanosomes at this instance, however, was not studied.

There were no marked differences between the use of immunosuppressed mice and *in vitro* system in the time taken to attain high resistance levels. Similar induction periods have been achieved by previous workers, in mice (Osman, *et al.*, 1992, Pospichal *et al.*, 1994) and in culture (Ross and Barns, 1996).

It was found from all the three induction methods that the most crucial stages were the initial steps, requiring very small incremental doses. Once these stages were accomplished the parasites rapidly gained resistance. Higher stepwise increases were then possible and relapses occurred within relatively short time periods. Similar observations have been reported on induction of mel Cy, diminazene aceturate and isometamidium resistance in *T. evansi* using immunosuppressed mice (Osman *et al.*, 1992).

This initial difficulty in the rate of development of resistance followed by an apparent "breakthrough" resulting in rapid increase in resistance has not been explained. This may have to do with the mechanism of selection. It has been suggested that resistance is the result of mutational events occurring at low frequency in individual trypanosomes which are usually eliminated by antibodies in immunocompetent host following treatment (Osman *et al.*, 1992). These mutants tend to survive and benefit from natural selection in immunosuppressed hosts. Lower drug doses may ensure the preservation of this minute segment of the population in immunosuppressed hosts. As induction progresses there is a build up of resistant mutants. With time these would replace the more susceptible population resulting in rapid responses to higher doses of drug, as seen in this study.

In contrast to the successful development of resistance in immunosuppressed mice it was extremely difficult to induce high resistance to mel Cy in immunocompetent mice. Osman *et al.* (1992) previously reported unsuccessful attempts to develop mel Cy and diminazene resistance in immunocompetent mice. These authors could not induce resistance beyond 0.5 mg/kg using either mel Cy or diminazene. In this study, resistance was induced up to 1.4 mg/kg in immunocompetent mice.

Osman *et al.* (1992) and Pospichal *et al.* (1994) previously induced mel Cy resistance using immunosuppressed mice. Zhang *et al.* (1993), however, reported successful induction of mel Cy (and diminazene) resistance in *T. evansi* using immunocompetent mice. In this study Tyler's original (TO) mice were used for induction of resistance. However, both Osman *et al.* (1992) and Zhang *et al.* (1993) used the same type of mice (Swiss white) in their studies. Therefore failure to develop resistance in immunocompetent mice observed in this study can not be attributed to strain variations in host responses to therapy.

This study highlights the importance of the role played by host immunity in the chemotherapy of parasitic infections. It shows that immunosuppression of the host considerably reduces the efficacy of antitrypanosomal drugs and can lead to rapid development of high levels of drug resistance.

Previously the part played by the host in the development of drug resistance was not recognised (Doenhoff *et al.*, 1991). It was thought that the host provided a passive medium in which trypanosomes and drugs interacted without interference. Innovations of methods to immunocompromise the host prior to infection such as splenectomy (Jansco and Jansco, 1935) led to increased understanding of this concept. This method was found to reduce the efficacy of suramin, and led to rapid development of suramin resistance in experimentally immunosuppressed animals.

The rapid development of drug resistance in immunosuppressed hosts has serious implications in trypanosomosis as this disease is known to adversely affect the host immune system. Besides, in endemic areas animals at risk of trypanosomosis are often found with other protozoan infections such as babesiosis and toxoplasmosis. These may induce various degrees of immunosuppression in affected hosts. Besides infection with immunosuppressive pathogens, other forms of stress including intercurrent infections with helminths (Fakae and Chiejina, 1993), poor nutrition, or even pregnancy may constitute indirect ways by which the natural resistance of trypanosome-infected animals is compromised. Results from this study suggest that such situations would encourage the development and spread of drug resistant trypanosomes.

The immune dependence of chemotherapy is known to occur in a variety of other parasitic and microbial infections in both man and animals (see Doenhoff *et al.* 1991). Early experimental evidence of the inter-relationship between drugs and immunity were observed in chicken malaria in which it was found that acquired immunity played a key role in achievement of cure with quinine treatment. Similar observations in *S. mansoni* showed that several drugs killed fewer adult worms in immunosuppressed mice (Sabah *et al.*, 1985). The efficacy of antimony, oxamniquine and praziquantel was found to be increased by concurrent passive transfer of immune serum with administration of drug. Similarly, the efficacy of antileishmanial drug, sodium stibogluconate, against *L. donovani* infections have been shown to be considerably reduced in immunosuppressed mice (Iwobi *et al.* 1991).

Among the drugs used against animal trypanosomosis the efficacy of quinapyramine has been reported to depend on a fully functional host immunity (Sen *et al.*, 1955). The prophylactic properties of homidium bromide are thought to be dependent on the stimulation of the host immune mechanisms after initial clearance of infection by the drug (Gilbert and Newton, 1982). In human trypanosomosis the efficacy of mel B treatment in mice has been shown to be reduced by immunosuppression (Frommel 1988), and the efficacy of difluoromethyl ornithine (DFMO) has also been shown to depend on the supporting role of antibodies (De Gee *et al.*, 1983).

Results from this study suggest that a population of trypanosomes naturally resistant to the selection dose of mel Cy was not originally present in the unselected isolate. These may have been formed during the induction experiment by adaptation and subsequent selection. Similar findings were made by Pospichal *et al.* (1994). These authors further confirmed this hypothesis by demonstrating that an intermediate population of *T. brucei* obtained during induction showed intermediate sensitivity between the original unselected clone and the drug resistant clone.

The results of *in vitro* drug sensitivity assays for mel Cy agreed well with the *in vivo* sensitivity tests. For example the 2-fold decrease in minimum curative (MCD_{100}) in the culture adapted WTAX parasites was reflected in a 1.7-fold decrease in IC_{50} values *in vitro*.

The variability of IC_{50} for mel Cy between the drug resistant lines indicates that they were at different levels of resistance *in vivo*. This show the limitation of *in vivo* methods in detecting higher levels of resistance. In this case responses could not be measured beyond 40 mg/kg mel Cy due to the toxicity of this drug in mice beyond this dose.

The mel Cy resistance induced in immunosuppressed mice and in culture both appeared stable after a single passage in immuncompetent mice. However, after several passages in the absence of drug pressure it was found that the resistance developed *in vivo* was not stable. It is possible that with further passages in mice in the absence of drug resistance may be ultimately lost. This finding differs from some other observations on the stability of resistance to mel Cy. Osman *et al.*, (1992)

reported that resistance to mel Cy induced in *T. evansi* using irradiated mice was stable after a single passage through immunocompetent mice. This is in agreement with the finding in this study. Scott *et al.* (1996) however, found that passaging of two mel Cy resistant *T. brucei* lines after removal of drug pressure had no detectable effect on levels of drug resistance. Those mel Cy resistant lines were also successfully transmitted through *Glossina*, undergoing complete cyclical development with no loss in levels of resistance. Stability of mel Cy induced resistance after cyclical transmission through tsetse has also been reported by Pospichal *et al.* (1994).

In contrast the mel Cy resistance induced *in vitro* in this study was found to be stable. This shows that the two induction systems selected for different resistance phenotypes, one exhibiting stable resistance and the other unstable. Reports from field workers suggest that drug resistance may or may not be stable (Harinasuta and Bunnag, 1988). It is thought that back mutations occurring after withdrawal of drug could result to reduction or loss of resistance (Harinasuta and Bunnag, 1988).

Cross resistance between mel Cy and mel B observed in this study is expected since both compounds are trivalent melaminophenyl arsenicals. Other workers have also reported cross resistance between melaminophenyl arsenicals. Fairlamb *et al.* (1992a) reported high levels of cross resistance between sodium melarsen, a pentavalent melaminophenyl arsenical and the trivalent arsenicals, melarsen oxide, melarsoprol and trimelarsen, in *T. brucei*. However, no cross resistance was expressed to phenylarsine oxide which lacks the melamine ring. Similarly 14-fold reduction in sensitivity to mel B in mel Cy resistant *T. brucei* was demonstrated by Pospichal *et al.* (1994). Ross and Barns (1996) showed cross resistance between mel Cy and mel B in *T. evansi*.

Cross resistance between melaminophenyl arsenicals suggests common mechanisms of uptake. It has been proposed that melaminophenyl arsenicals are taken up by trypanosomes via a common purine transporter. This transporter was found to be altered in an arsenical-resistant line of *T. brucei* (Carter and Fairlamb, 1993). It

would be interesting to determine if the resistant lines produced in the present study also possess altered purine transport systems.

High levels of cross- resistance to Berenil® were also observed in mel Cy resistant lines produced in this study. Cross resistance between diamidines and melaminophenyl arsenicals have been observed previously by a number of workers: High levels of cross resistance to diminazene aceturate and pentamidine *in vivo* in mel Cy resistant *T. evansi* was reported by Osman, *et al.* (1992). Fairlamb *et al.* (1992a) reported varying degrees of cross resistance to stilbamidine (38-fold), Berenil® (31.5-fold), propamidine (5.7-fold) and pentamidine (1.5-fold), in *T. brucei* selected using a pentavalent melaminophenyl arsenical, sodium melarsen. Pospichal *et al.* (1994) also found cross resistance to 2 diamidines: diminazene aceturate (47-fold) and pentamidine methanosulphonate (34-fold), in *T. evansi* made resistant to mel Cy. Ross and Barns (1996) also reported cross resistance between mel Cy and Berenil®. Frommel and Balber (1987) selected drug resistant clones of *T. brucei* using melarsoprol (mel B) and found that these were cross resistant to Berenil®.

Zhang et al. (1993) reported the induction of resistance to mel Cy in *T. evansi* which became cross resistant to both Berenil® and pentamidine. These authors found that the sensitivity of Berenil®-resistant clones to mel Cy varied: Two *T. evansi* clones and one *T. equiperdum* clone which were Berenil® resistant remained very sensitive to mel Cy. In contrast another Berenil®-resistant *T. evansi* clone became at least 128-fold more resistant to mel Cy than its parental clone. It was suggested that Berenil®-resistant clones displayed two different types of resistance: a single drug resistance demonstrated by clones which remained sensitive to mel Cy, and cross resistance , illustrated by the clone which showed cross resistance to mel Cy.

This study show that mel Cy-resistant *T. evansi* possess only one type of resistance mechanism which displays cross resistance to the diamidines.

It is suggested that resistance to arsenical drugs and the diamidines is based on altered drug transport (Bacchi, 1993). Carter and Fairlamb (1993) suggested that the melaminophenyl arsenicals as well as the diamidines are taken up on a common

transporter. The common feature of recognition is thought to reside in the melamine and benzamidine moieties, respectively (Fairlamb *et al.* (1992b).

Cross resistance between melaminophenyl arsenicals and quinapyramine is not common. There are conflicting reports on the occurrence of cross resistance between this group of arsenicals and quinapyramine. Ross and Barns (1996) found that induction of resistance to mel Cy in a *T. evansi* strain *in vitro* did not result to increased resistance to quinapyramine. Pospichal *et al.* (1994), on the other hand reported high level of cross resistance (40-fold) to quinapyramine in procyclic forms of *T. brucei* with low resistance to mel Cy. Zhang *et al.* (1993), found clones of *T. evansi* selected for resistance to mel Cy, Berenil® and suramin to be more sensitive to quinapyramine *in vivo* than the parent clone.

No cross resistance between mel Cy and suramin was found in this study in agreement with previous reports (Zhang *et al.* 1993; Pospichal *et al.* 1994; Ross and Barns 1996). Cross resistance between arsenicals and suramin have not been found in field isolates (Ross and Barns, 1996). Fairlamb *et al.* (1992a) reported a low level of cross resistance (5.8 -fold) to suramin in melarsen-resistant *T. brucei*. Very little is known about resistance mechanisms to suramin as this drug is known to have several drug targets in the parasite.

It was found in this study that moderate to high increases in sensitivity to trypanocides occurred when *T. evansi* TREU 1840 was adapted to *in vitro* growth. It is likely that adaptation to *in vitro* culture resulted to some modification of the sensitivities of this isolate especially to quinapyramine and suramin, resulting to increased sensitivity to the two drugs. This observation differs from that of Brown *et al.* (1987) who found that adaptation of *T. congolense* stocks to *in vitro* culture did not change their sensitivities to isometamidium.

The results of the *in vitro* drug sensitivity assays with mel Cy were found to correlate well with the *in vivo* drug sensitivity tests. This shows that this *in vitro* assay is suitable for detecting drug resistance.

CHAPTER FIVE

ARSENICAL-INDUCED *IN VITRO* LYSIS OF DRUG-SENSITIVE AND DRUG-RESISTANT *TRYPANOSOMA EVANSI*

5.1. INTRODUCTION

Bloodstream forms of trypanosomes are known to be extremely sensitive to trivalent aromatic arsenicals. Upon exposure to drug *in vitro* the parasites rapidly lose motility and undergo lysis (Clarkson and Amole, 1982; Fairlamb *et al.*, 1989). Loss of motility was attributed to inhibition of critical enzymes of glycolysis. Arsenicals inhibit fructose-6-phosphate 2-kinase, an enzyme that produces the glycolytic intermediate, fructose-2, 6-bisphosphate. Fructose-2, 6-bisphosphate is a specific activator of pyruvate kinase in the strictly glycolytic long slender trypanosome. This slows down glycolysis and significantly reduces ATP production (Van Schaftingen *et al.*, 1987).

It is suggested that a unique dithiol-containing dipeptide, dihydrotrypanothione (N^1, N^8-bis (gluthathionyl) spermidine) is a primary target for arsenical drugs. The arsenic moiety of the arsenicals is thought to combine rapidly within the cell with the -SH groups on trypanothione to form a stable arsenical-trypanothione complex, Mel T. Mel T secondarily acts as a competitive inhibitor of trypanothione reductase, a flavoprotein disulphide oxidoreductase (Fairlamb, *et al.*, 1989). Trypanothione reductase together with dihydrotrypanothione is essential for the regulation of the correct intracellular redox balance. These protect trypanosomes from oxidant damage (Fairlamb *et al.*, 1992b). Fairlamb *et al.* (1992b) showed that trivalent arsenicals irreversibly inhibit trypanothione reductase *in vitro.* The depletion of free trypanothione and the blockage in the recycling of oxidized trypanothione lead to increased oxidant stress. This is partially responsible for trypanosome lysis caused by arsenicals (Fairlamb, *et al.*, 1989; Yarlett *et al.*, 1991).

Studies on the mechanisms of resistance to arsenical drugs (Fairlamb *et al.*, 1992b) have shown that resistance is not due to overproduction of the primary or secondary

drug targets. Other studies by Yarlett *et al.* (1991) on *T. brucei* failed to demonstrate inactivation of drug as a mechanism of resistance. More recent studies (Damper and Patton, 1976; Carter and Fairlamb, 1993; Ross and Barns, 1996), suggest that normal trypanosomes concentrate trivalent arsenicals more efficiently than arsenical resistant organisms. Toward investigation of decreased uptake as a mechanism of resistance development it is necessary to establish the mode of uptake or entry of arsenical drugs into trypanosomes.

Besides the need for methods for rapid assessment of arsenical susceptibility of trypanosomes, *in vitro* lysis by arsenicals (Yarlett *et al.* (1991) can be utilised to explore mechanisms of arsenical uptake into trypanosomes. Based on the observation that melamine antagonises the trypanocidal activity of melaminophenyl arsenicals, Carter and Fairlamb (1993) utilised the trypanolytic effect of melarsen oxide, to determine other compounds that compete for the same receptor. This study showed for the first time that melarsen oxide was taken up by an adenosine transporter.

This study aims to investigate further the involvement of adenosine transport in the uptake of melaminophenyl arsenicals, in *T. evansi*. This will be achieved by the determination of the interaction between two arsenical drugs (mel Cy and mel B,) and adenosine transport system in *T. evansi*, using an *in vitro* spectrophotometric lysis assay. The specific aims considered in this chapter include the following:

1. To ascertain whether the arsenicals (mel Cy and mel B) cause lysis of *T. evansi* similar to that observed with other arsenical drugs in related species or strains.

2. Determine to what extent *in vitro* lysis assay can differentiate between mel Cy-sensitive and mel Cy-resistant trypanosomes.

3. Measure cross resistance between mel Cy and other melaminophenyl arsenicals using *in vitro* lysis assay.

4. Identify which compounds inhibit lysis of *T. evansi* by mel Cy and mel B with a view to establishing the mode of uptake into the parasite.

5. Compare the response to *in vitro* lysis of the *in vivo*-and *in vitro*-derived drug resistant *T. evansi.*

5.2. MATERIALS AND METHODS

5.2.1. Trypanosomes

Trypanosomes derived using both the *in vivo* and *in vitro* systems were used in this study. The mouse-maintained lines include the drug sensitive isolate (WT) and its drug resistant derivatives (CR2, CR3, CR3.1, CR_{9d}, and CR_{11d}). The CR_{9d} and CR_{11d} were intermediate stabilates prepared from sub passages of CR2 at 40 mg/kg mel Cy. CR_{9d} and CR_{11d} were passaged 9 and 11 times, respectively. Parasites were still growing in the presence of the drug. The culture-adapted lines included the drug sensitive isolate (WTAX) and its drug resistant derivative (MCR1.1).

5.2.2. Arsenical drugs

Two trivalent melaminophenyl arsenical drugs, mel Cy and mel B, were used in the lysis experiments. Their sources have been described in the General Methods (see section 3.3). Mel Cy was prepared and diluted to appropriate concentrations fresh in distilled deionised water. This was used once on the day of the experiment. Mel B was diluted in propylene glycol and stored in aliquots at 4°C for up to 4 weeks.

5.2.3. Other chemicals

Inhibitors including adenosine, adenine, inosine and Berenil® were obtained and stored as described in the General Methods (section 3.3). Stock concentrations of adenosine, adenine and inosine were prepared by dissolving in 25 mM NaOH. Dilutions were made in PSG and stored in aliquots of 0.5 ml at -20°C. These were thawed once and used on the day of the experiment. Diminazene (Berenil®) was prepared fresh in double distilled water and also used once on the day of the experiment. The amount of diminazene was calculated as 45% content of Berenil®.

5.2.4. Lysis assays

Trypanosomes either separated from mouse blood or harvested from culture supernatant were washed by centrifugation at 1000x g for 10 minutes. The pellet was suspended in 1.5 ml of PSG in an Eppendorf tube, and washed two times by

centrifugation at 100x g for 6 minutes in a biofuge. The pellet was resuspended in PSG at a density of 10^8 trypanosomes/ml. Trypanosomes separated from mouse blood were kept on ice.

Nine hundred µl of lysis buffer (Carter's Balanced Salt Solution, CBSS, (Fairlamb *et al.*, 1992a), consisting of 25 mM Hepes/120 mM NaCl/5.4 mM KCl/0.55 mM CaCl$_2$/0.4 mM MgSO$_4$/5.6 mM Na$_2$HPO$_4$/11.1 mM glucose, pH adjusted to 7.4 with NaOH; were added to spectrophotometer cuvettes and kept at 37°C on a water bath. Trypanosome suspensions were prewarmed to 37°C for 5 minutes. Aliquots of 100 µl cells, containing 10^7 trypanosomes were added to each incubating tube. Inhibitors prepared at 100x the required concentration in assay were added in 10 µl volumes. Arsenical drugs were prepared at 50x final concentration in assay. 20 µl amount were added at 20 seconds intervals to incubation mixture, at the start of the experiment. Readings were taken at zero time (t_0) when the arsenical was added. Thereafter lysis was measured at 5 minutes intervals in a spectrophotometer, for 30 minutes. As the trypanosomes were lysed by the arsenical there was decrease in turbidity and more light was transmitted. Decrease in absorbance was recorded at a wavelength of 750 ηm in a spectrophotometer.

Control incubations consisting of trypanosomes in lysis buffer each received 20 µl of solvent instead of arsenical drug. A second control consisting of trypanosomes plus arsenical drug and no inhibitor was included in inhibition experiments. Each experiment was repeated at least 2 separate times.

The effect of increasing concentrations of adenosine, adenine, inosine and Berenil® on arsenical induced lysis of the drug sensitive lines (WT, WTAX) was investigated.

5.3. RESULTS

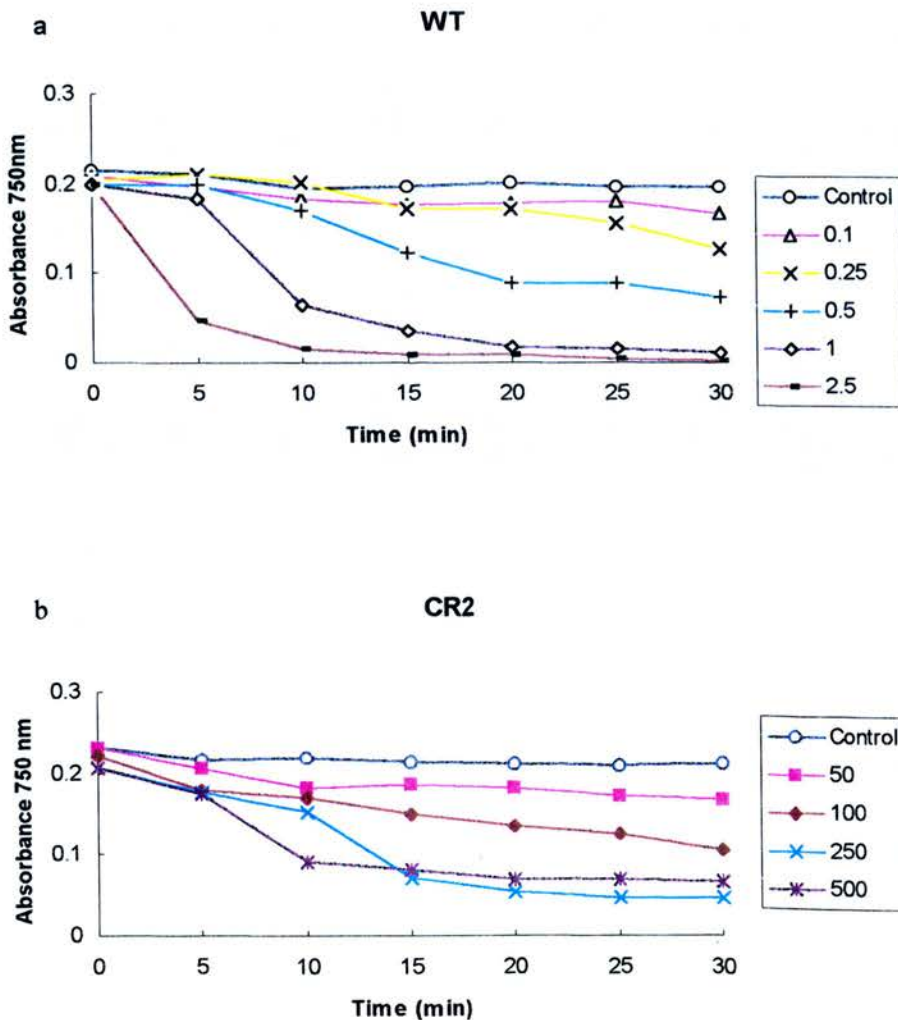### 5.3.1. *In vitro* lysis of mouse-adapted *Trypanosoma evansi* lines by mel Cy.

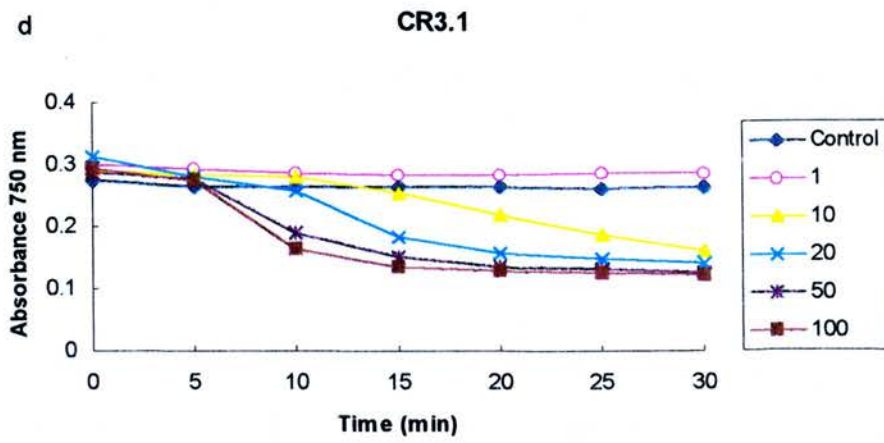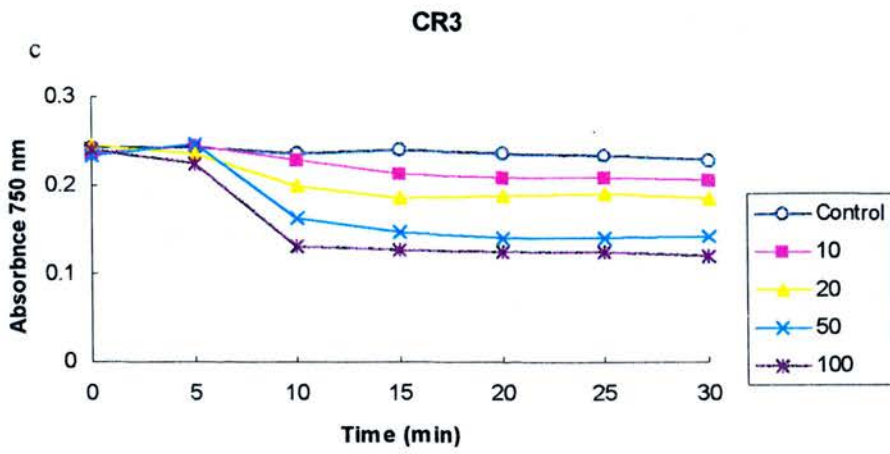#### 5.3.1.1. Drug sensitive trypanosomes

The lysis of drug sensitive *T. evansi* (WT) is shown in Figure 5.1a. Mel Cy caused lysis of drug sensitive trypanosomes in a concentration and time dependent manner. Little or no measurable lysis occurred at 0.1 µM mel Cy. Absorbance curves at this dose were similar to control with no added drug. At 0.25 µM lysis was detectable after 10 minutes incubation with the drug. Appreciable lysis occurred at 0.5 µM after 5 minutes incubation. Rapid lysis occurred at 1.0 µM mel Cy, but the 5 minutes delay in onset was still maintained. Immediate and rapid lysis was observed at concentrations of 2.5 µM and above.

5.3.1.2. In vivo-derived drug resistant trypanosomes

Lysis of the *in vivo*-derived drug resistant trypanosome lines (CR2, CR3 and CR3.1) are shown in Figures 5.1b-d. There was variable degree of lysis in each of the drug resistant population. All the three resistant lines were not lysed by 1.0 µM concentration of mel Cy (Figure 1d). This showed that there was a definite increase in resistance. Partial lysis was observed with 10-50 µM mel Cy in all the resistant lines (Figures 1b, c and d). Partial lysis also varied between the different resistant lines, being least in the CR2 and greatest in the CR3.1. The extent of this partial lysis was comparable to the lysis at 0.25-0.5 µM in the drug sensitive line, and may suggest a 100- fold increase in tolerance to the drug *in vitro*. More lysis occurred between 100 and 500 µM mel Cy in these resistant lines. However, lysis was not rapid even at these high concentrations of mel Cy, and absorbance values never approached zero.

Figure 5.1: *In vitro* lysis of the mouse-maintained *Trypanosoma evansi* by increasing concentrations of mel Cy. a, drug sensitive isolate (WT); b, c and d, *in vivo*-derived drug resistant lines (CR2, CR3, CR3.1). 1×10^7 trypanosomes prewarmed to 37°C were incubated in 1 ml of lysis buffer in spectrophotometer cuvettes. Varying concentrations of mel Cy were added to each incubation mixture at the start of the experiment. Absorbance due to presence of live trypanosomes was measured at time zero (t_0) when the arsenical was added. Thereafter lysis was measured at 5 minutes intervals for 30 minutes. As the trypanosomes were lysed by the arsenical there was decrease in turbidity and more light was transmitted. Decrease in absorbance was recorded at a wavelength of 570 ηm in a spectrophotometer. Control incubations with trypanosomes and no arsenical were included in each experiment. Each experiment was repeated at least 3 separate times and similar results were obtained. Doses are in μM mel Cy.

CR3

c

CR3.1

d

5.3.2. *In vitro* lysis of culture-adapted *Trypanosoma evansi* by mel Cy

5.3.2.1. Lysis of drug sensitive trypanosome cultures

Figure 5.2a shows lysis of culture-adapted, unselected *T. evansi* (WTAX) by mel Cy. Lysis was largely similar to that observed in the mouse-maintained unselected line (WT). However, slightly more lysis was observed at 0.25 and 0.5 μM concentrations compared with the WT parasites. Although rapid lysis occurred at 1.0 μM lysis at doses of 2.5 and 5.0 μM were less rapid compared to lysis at equivalent doses in the WT.

5.3.2.2. Lysis of in vitro-derived drug resistant trypanosomes

Lysis of the *in vitro*-derived drug resistant *T. evansi* (MCR1.1) is shown in Figure 5.2b. High resistance to *in vitro* lysis was shown by these parasites as opposed to the *in vivo*-derived lines. Trypanosomes were not lysed by 50 μM mel Cy, representing over 200-fold increase in resistance to mel Cy compared to WTAX. Partial lysis occurred at 100 μM. Increased lysis at 250 μM was only similar to lysis at 0.25 μM in the drug sensitive parasites, and lysis was not complete at 500 μM concentration.

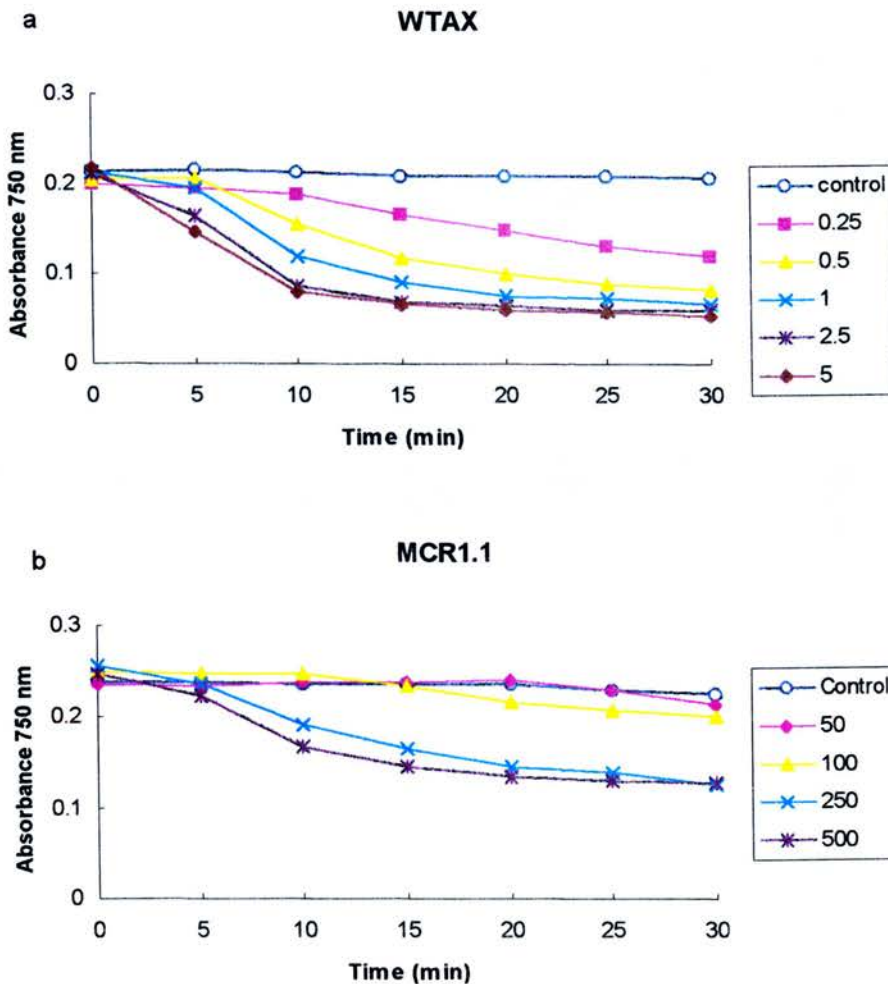### 5.3.3. Inhibition of mel Cy lysis in *Trypanosoma evansi*

5.3.3.1. Inhibition of mel Cy lysis in mouse-adapted Trypanosoma evansi

Effect of inhibition of lysis by mel Cy of drug sensitive *T. evansi* maintained in mice was investigated using purine compounds such as adenine, adenosine and inosine; and a diamidine drug, Berenil®. The results of inhibition studies are shown in Figures 5.3 a-d.

Figure 5.2: *In vitro* lysis of the culture-adapted *Trypanosoma evansi* by increasing concentrations of mel Cy. a, drug sensitive isolate (WTAX); b, *in vitro*-derived drug resistant line (MCR1.1). 1×10^7 trypanosomes prewarmed to 37°C were incubated in 1 ml of lysis buffer in spectrophotometer cuvettes. Varying concentrations (μM) of mel Cy were added to each incubation mixture at the start of the experiment. Absorbance due to presence of live trypanosomes was measured at time zero (t_0) when the arsenical was added. Thereafter lysis was measured at 5 minutes intervals for 30 minutes. As the trypanosomes were lysed by the arsenical there was decrease in turbidity and more light was transmitted. Decrease in absorbance was recorded at a wavelength of 750 ηm in a spectrophotometer. Control incubations with trypanosomes and no arsenical were included in each experiment. Each experiment was repeated at least 3 separate times and similar results were obtained. Doses are in μM mel Cy.

5.33.1.1. Inhibition of mel Cy lysis by adenosine

Lysis of the drug sensitive *T. evansi* (WT) by 1 µM mel Cy was inhibited in a concentration and time dependent manner by adenosine (Figure 5.3a). Adenosine concentrations in 1 and 2x excess (1 and 2 µM) of mel Cy had very little or no effect on mel Cy lysis. However 5x excess (5 µM) concentration delayed lysis up to 10 minutes. Thereafter only about 50% lysis occurred compared to control incubations with 1.0 µM mel Cy. Increased inhibition of lysis was observed at 10x excess adenosine and lysis was delayed up to 15 minutes. At 50x excess (50 µM) adenosine protected trypanosomes from mel Cy lysis up to 20 minutes, and less than 20% lysis occurred. Maximum protection was obtained at 100x excess (100 µM) concentration. At this concentration lysis was delayed up to 20 minutes and over 90% protection was achieved.

5.3.3.1.2. Inhibition of mel Cy lysis by adenine

Adenine inhibited lysis of *T. evansi* by mel Cy in a similar manner as adenosine (Figure 5.3b). However adenine appeared to be more potent at inhibition compared to adenosine. Some inhibition of lysis was observed even at 1 and 2x excess (1 and 2 µM) concentrations of adenine. Similarly increased protection was observed at 5 and 10x excess (5 and 10 µM) concentrations of adenine. Lysis was delayed up to 15 and 20 minutes, respectively. At 50x excess (50 µM) lysis was delayed for 25 minutes and approximately 95% protection was achieved. Complete inhibition of lysis was observed at 100x excess (100 µM) concentration of adenine and no lysis was detected after 30 minutes incubation with the drug.

5.3.3.1.3. Lack of inhibition of mel Cy lysis by inosine

No measurable inhibition or delay of lysis by 1 µM mel Cy for 30 minutes was observed with up to 100x excess (100 µM) concentrations of inosine (Figure 5.3c).

Figure 5.3: Inhibition of mel Cy lysis in the mouse-maintained *Trypanosoma evansi* (WT) by increasing concentrations of inhibitors. a, adenosine; b, adenine, c, inosine, and, d, Berenil®. 1×10^7 trypanosomes separated from mice blood were prewarmed to 37°C and incubated in 1 ml of lysis buffer at 37°C. Inhibitors prepared at 100x final concentrations in assay were added in 10 μl volumes to incubation mixtures. Mel Cy (MC) was prepared at 50 times final concentration of 1 μM. 20 μl were added to each incubation mixture at the start of the experiment. Absorbance due to presence of trypanosomes was measured at zero time (t_0) when the arsenical was added. Blocking of mel Cy lysis by inhibitor represented by slowing down in the rate of decrease in turbidity, was measured at 5 minutes intervals for 30 minutes. Absorbance was recorded in a spectrophotometer at a wavelength of 750 ηm. Two control incubations were included in each experiment: one with trypanosomes and no arsenical and the second with trypanosomes and 1 μM mel Cy but no inhibitors. Each experiment was repeated at least 3 separate times and similar results obtained.

"x" represents number of times inhibitor concentration is in excess of mel Cy concentration of 1 μM.
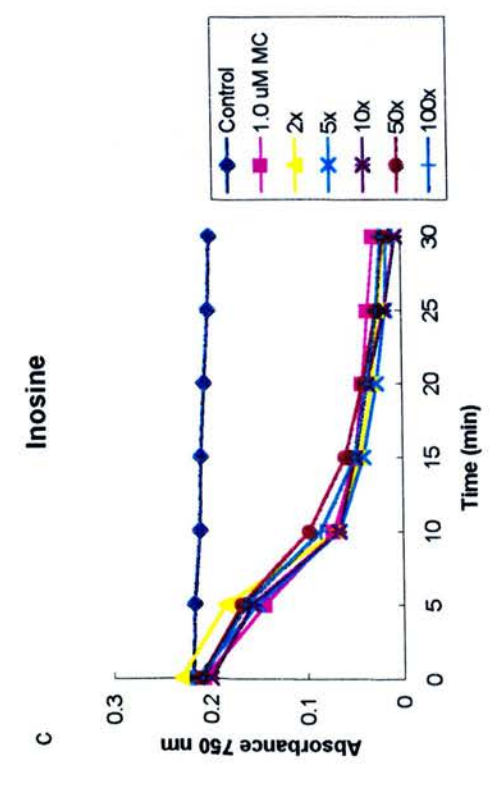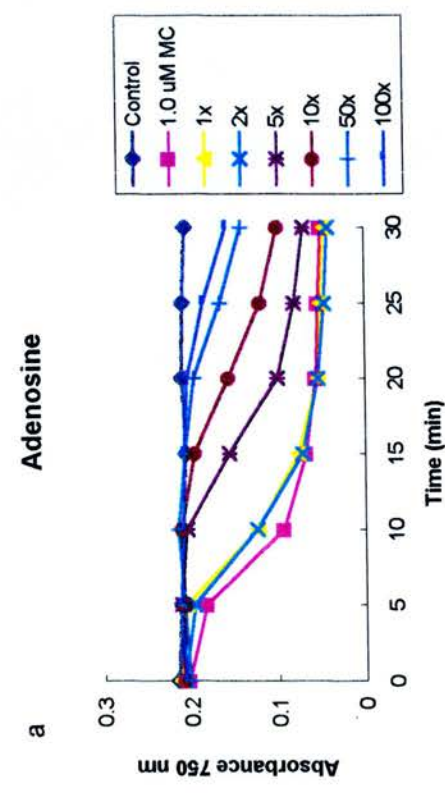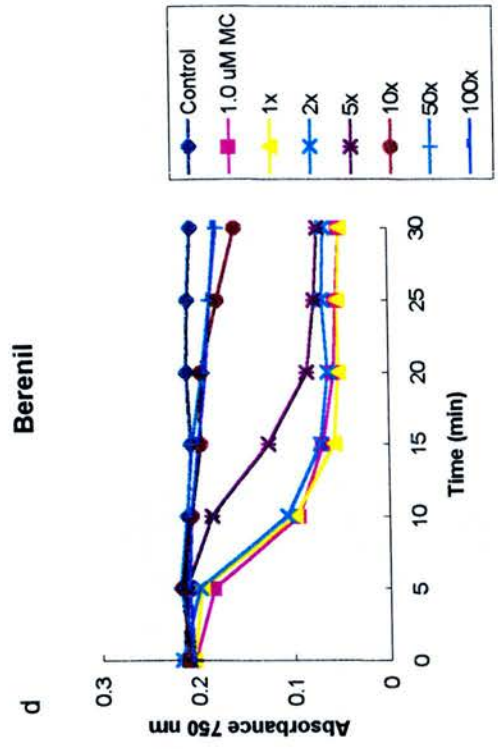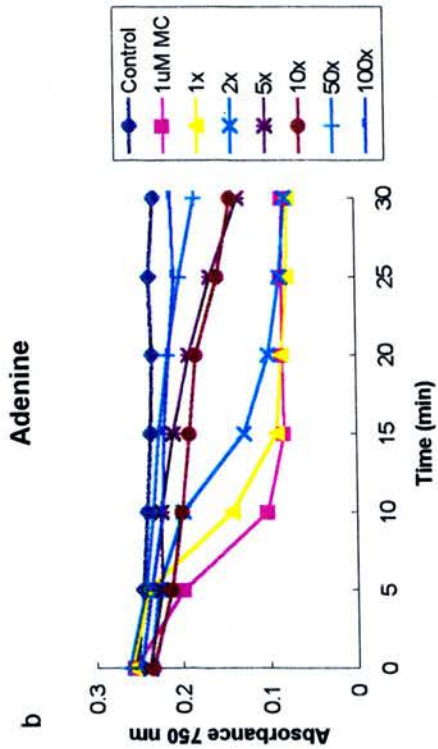
5.3.3.1.4. Inhibition of mel Cy lysis by Berenil®

A dose-dependent inhibition of lysis by 1 μM mel Cy was observed with Berenil®, similar to adenine and adenosine (Figure 5.3d). No inhibition of lysis was observed with up to 2x excess (2 μM) Berenil®, but inhibition occurred at 5x excess (5 μM) concentration. Markedly enhanced inhibition (approximately 95%) was observed at 10x excess (100 μM) concentration of Berenil®, compared to the same concentration of adenine and adenosine. At 50x excess (50 μM) there was complete (100%) inhibition of lysis.

5.3.3.2. Inhibition of mel Cy lysis in culture-adapted trypanosomes by adenosine

Comparative studies on inhibition of lysis by mel Cy were done on the culture-adapted *T. evansi* using adenosine. Similar to the mouse-maintained trypanosomes adenosine inhibited lysis by 1 μM mel Cy in the WTAX trypanosomes in a dose-dependent manner (Figure 5.4). Little inhibition occurred at 1x excess concentration. In these trypanosomes, 50x excess concentrations of adenosine conferred 100% protection from lysis by 1 μM mel Cy.

5.3.4. *In vitro* lysis of mouse-maintained *Trypanosoma evansi* by mel B

5.3.4.1. Lysis of drug sensitive trypanosomes

Results of mel B lysis of the drug sensitive *T. evansi* maintained in mice (WT) are shown in Figure 5.5a. Similar to mel Cy, mel B caused lysis of drug sensitive trypanosomes in a concentration and time dependent manner. However, mel B appeared to be less effective in lysing drug sensitive trypanosomes compared to mel Cy. 10-15 μM concentrations of mel B caused little lysis, similar to lysis by 0.1-0.25 μM mel Cy in the same parasites. Rapid lysis occurred at 20 μM mel B. This was equivalent to 0.5 μM mel Cy lysis. 50 μM mel B caused rapid lysis comparable to 1.0 μM mel Cy in these trypanosomes, being over 50 times excess of mel Cy. Lysis was delayed at least 5 minutes regardless of concentration of mel B used.
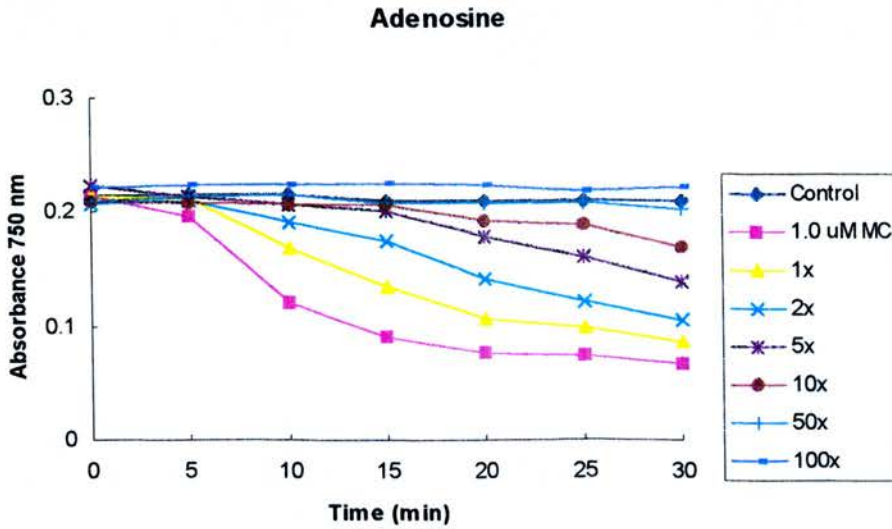
Figure 5.4: Inhibition of mel Cy lysis in culture-adapted *Trypanosoma evansi* (WTAX) by increasing concentrations of adenosine. 1×10^7 trypanosomes separated from mice blood were prewarmed to 37°C and incubated in 1 ml of lysis buffer, at 37°C. Adenosine was prepared at 100x final concentrations in assay. 10 µl volumes were added to incubating trypanosomes. Mel Cy (MC) prepared at 50 times final concentration of 1 µM, was added in 20 µl volumes at the start of the experiment. Absorbance was first measured at zero time (t_0) when the arsenical was added. Blocking of mel Cy lysis by adenosine was measured at 5 minutes intervals for 30 minutes. Absorbance was recorded at 570 ηm in a spectrophotometer. Two controls were included in this experiment: one consisted of trypanosomes and no arsenical, and the second of trypanosomes and mel B but no inhibitor (adenosine). The experiment was repeated 2 separate times and similar results were obtained.

5.3.4.2. *Lysis of in vivo-derived drug resistant trypanosomes by mel B.*

Lysis of the *in vivo*-derived drug resistant trypanosomes (CR3.1) by mel B is shown in Figure 5.5b. Contrary to observations with the drug sensitive line, mel B was very efficient in lysing the drug resistant trypanosomes. No lysis was obtained at 10 µM concentration and very little lysis occurred with 20 µM. Pronounced lysis was observed at 50 µM mel B although lysis was delayed up to 10 minutes. Rapid lysis occurred at 100 and 250 µM concentrations in these trypanosomes. This was equivalent to lysis by 50 µM mel B in the drug sensitive parasites. A 5 minutes delay in lysis was maintained even at high concentrations of mel B.

5.3.5. *In vitro* lysis of culture-adapted *Trypanosoma evansi* by mel B

5.3.5.1. *Lysis of drug sensitive trypanosomes*

Lysis of the culture- adapted drug sensitive (WTAX) trypanosomes is shown in Figure 5.6a. Similar to the mouse-maintained parasites, mel B was less effective in lysing the cultured drug sensitive trypanosomes. No lysis was obtained in the presence of 5 µM mel B. However, more lysis was observed at 10 and 15 µM compared to WT parasites. Rapid lysis occurred at 20 µM and above concentrations of mel B. Lysis was complete at 40 µM mel B. There was delay in lysis for up to 5 minutes at concentrations of 10-20 µM, but immediate lysis was observed at 25 µM and above concentrations of mel B.

5.3.5.2. *Lysis of in vitro-derived drug resistant trypanosomes by mel B*

Lysis of the *in vitro*-derived drug resistant *T. evansi* (MCR1.1) is shown in Figure 5.6b. Little or no lysis was observed at 50 µM mel B. Some lysis occurred at 100 µM after 10 minutes delay. More lysis occurred at 250-500 µM. This represents a 2.5 fold increase in tolerance to mel B lysis in the drug resistant trypanosomes selected *in vitro*.
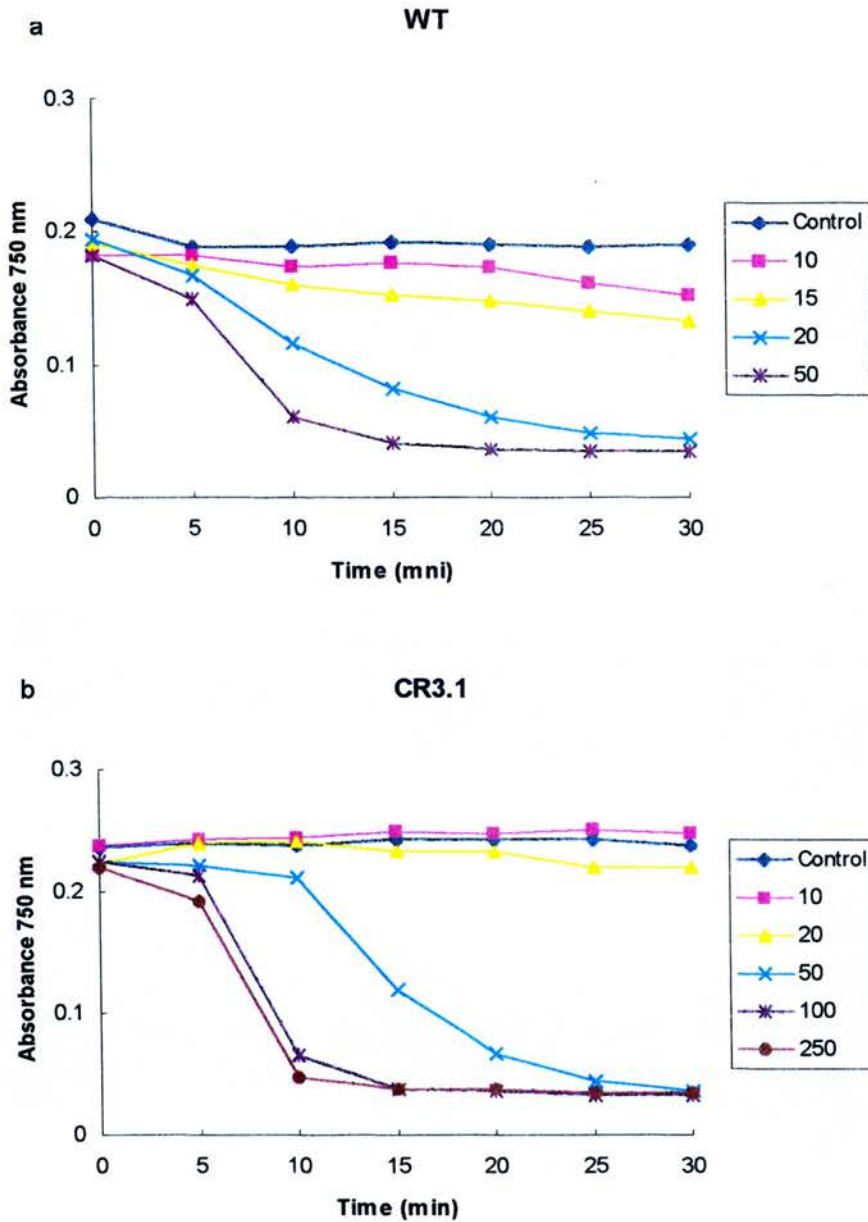
Figure 5.5: *In vitro* lysis of the mouse-maintained *Trypanosoma evansi* by increasing concentrations of mel B. a, drug sensitive isolate (WT); b, *in vivo*-derived drug resistant clone (CR3.1). $1x10^7$ trypanosomes were prewarmed and incubated in 1 ml of lysis buffer, at 37°C. Mel B prepared at 100x final concentrations in assay was added in 20 µl amounts to each incubation mixture at the start of the experiment. Absorbance due to presence of trypanosomes was measured at time zero (t_0) when the arsenical was added. Lysis represented by decrease in absorbance was measured at 5 minutes intervals for 30 minutes, at 750 ηm, in a spectrophotometer. Control incubations with trypanosomes and no arsenical were included in each experiment. Each experiment was repeated at least 3 separate times and similar results were obtained. Doses are in µM mel B.

a **WTAX**
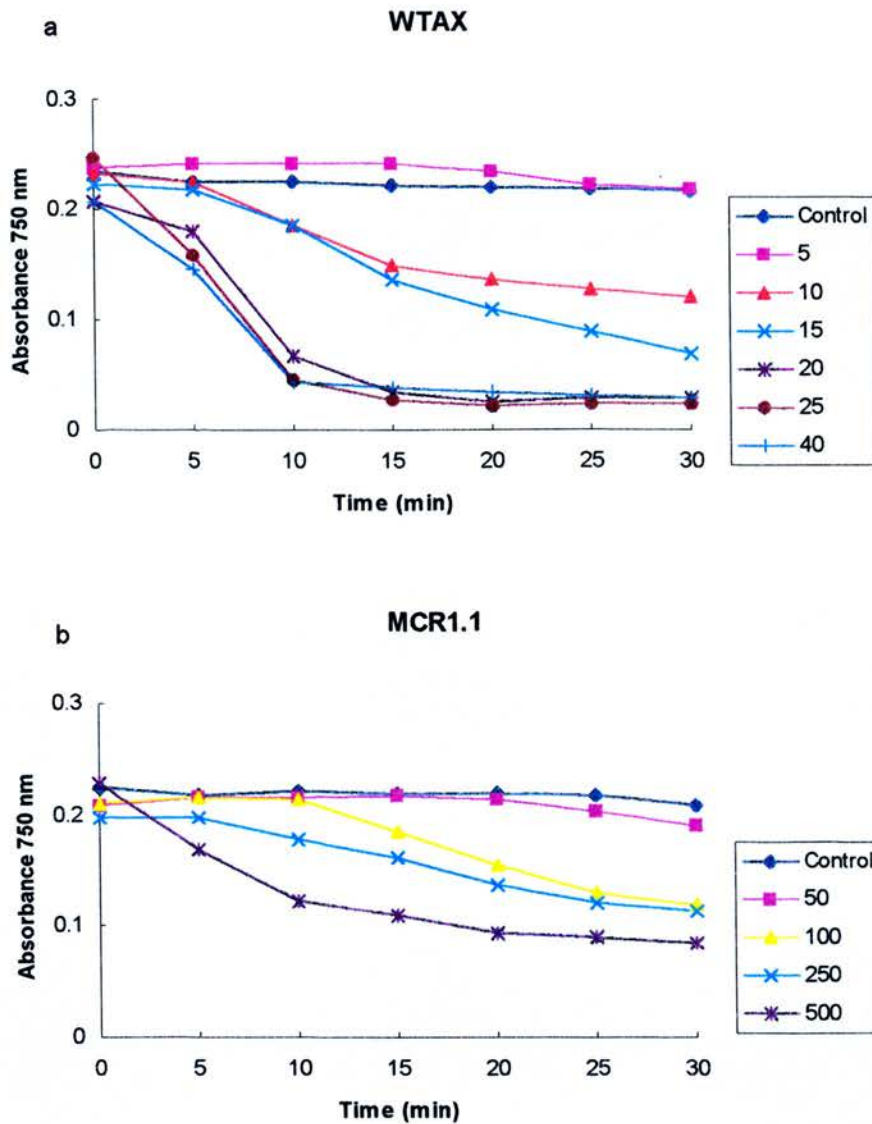
b **MCR1.1**

Figure 5.6: *In vitro* lysis of the culture-adapted *Trypanosoma evansi* by increasing concentrations of mel B. a, drug sensitive isolate (WTAX); b, *in vitro*-derived drug resistant clone (MCR1.1). 1×10^7 trypanosomes from cultures were prewarmed and incubated in 1 ml of lysis buffer at 37°C. Mel B prepared at 100x final concentrations in assay was added in 20 µl amounts to each incubation mixture, at the start of the experiment. Absorbance due to presence of trypanosomes was measured at time zero (t_0) when the arsenical was added. Lysis represented by decrease in absorbance was measured at 5 minutes intervals for 30 minutes, at 750 ηm in a spectrophotometer. Control incubations with trypanosomes and no arsenical were included in each experiment. Experiments were repeated at least 3 separate times and similar results were obtained. Doses are in µM mel B.

5.3.6. Inhibition of mel B lysis in the mouse-maintained *Trypanosoma evansi*

5.3.6.1. Lack of inhibition of mel B lysis by adenosine and adenine

No inhibition of lysis by 20 µM mel B was observed with the addition of up to 100x excess concentrations of either adenosine or adenine (Figure 5.7 a, b). This was in contrast to the observation on inhibition of mel Cy lysis by these compounds in the same parasites.

5.3.6.2. Lack of inhibition of mel B lysis by inosine.

Similar to observations on mel Cy lysis, no inhibition of lysis by 20 µM mel B was observed with up to 100x excess concentrations of inosine (Figure 5.7c).

5.3.6.3. Inhibition of mel B lysis by Berenil[®]

Results of inhibition of mel B lysis of WT trypanosomes by Berenil[®] are shown in Figure 5.7d. Concentration dependent inhibition of lysis was observed . No inhibition was observed at 1x excess (20 µM) concentration of Berenil[®], but pronounced inhibition occurred at 5 and 10x excess (100 and 200 µM). At 50x excess (1000 µM) Berenil[®], over 90% protection from lysis was observed. 100x excess (2000 µM) concentration of Berenil[®] completely inhibited lysis by 20 µM mel B.

5.3.7. Inhibition of mel B lysis of culture adapted *Trypanosoma evansi*

5.3.7.1. Lack of inhibition of mel B lysis by adenosine and adenine

Similar to the observations on the mouse-maintained *T. evansi* there was no inhibition of lysis by 20 µM mel B in the culture-adapted trypanosomes up to 100x excess (2000 µM) concentrations of either adenosine or adenine (Figures 5.8 a & b).

Figure 5.7: Inhibition of mel B lysis in the mouse-maintained *Trypanosoma evansi* (WT) by increasing concentrations of inhibitors. a, adenosine; b, adenine, c, inosine, and, d, Berenil. 1×10^7 trypanosomes separated from mouse blood were prewarmed to 37°C and incubated in 1 ml of lysis buffer at 37°C. Inhibitors prepared at 100x final concentrations in assay were added in 10 μl volumes to incubation mixtures. Mel B was prepared at 50 times final concentration of 20 μM, and 20 μl were added to each incubation mixture at the start of the experiment. Absorbance due to presence of live trypanosomes was measured at zero time (t_0) when the arsenical was added. Blocking of mel B lysis by inhibitor represented by slowing down in the rate of decrease in turbidity, was measured at 5 minutes intervals for 30 minutes. Absorbance was recorded in a spectrophotometer at a wavelength of 750 ηm. Two control incubations were included in each experiment: one with trypanosomes and no arsenical and the second with trypanosomes and 20 μM mel B but no inhibitors. Each experiment was repeated at least 3 separate times and similar results obtained. "x" represents number of times inhibitor concentration is in excess of mel B concentration of 20 μM.

Figure 5.8: Inhibition of mel B lysis in the culture-adapted *Trypanosoma evansi* (WTAX) by increasing concentrations of inhibitors. a, adenosine; b, adenine, c, inosine, and, d, Berenil® . 1×10^7 trypanosomes harvested from cultures were prewarmed to 37°C and incubated in 1 ml of lysis buffer at 37°C. Inhibitors prepared at 100x final concentrations in assay were added in 10 μl volumes to incubation mixtures. Mel B was prepared at 50 times final concentration of 20 μM, and 20 μl were added to each incubation mixture, at the start of the experiment. Absorbance due to presence of live trypanosomes was measured at zero time (t_0) when the arsenical was added. Blocking of mel B lysis by inhibitor represented by slowing down in the rate of decrease in turbidity, was measured at 5 minutes intervals, for 30 minutes. Absorbance was recorded in a spectrophotometer at a wavelength of 750 ηm. Two control incubations were included in each experiment: one with trypanosomes and no arsenical and the second with trypanosomes and 20 μM mel B but no inhibitors. Each experiment was repeated at least 3 separate times and similar results obtained. "x" represents number of times inhibitor concentration is in excess of mel B concentration of 20 μM.

a. Adenosine

b. Adenine

c. Inosine

d. Berenil

5.3.7.2. Lack of inhibition of mel B lysis by inosine

No inhibition of lysis by 20 μM mel B was observed with up to 100x excess (2000 μM) concentrations of inosine (Figure 5.8c), similar to observations on mel Cy.

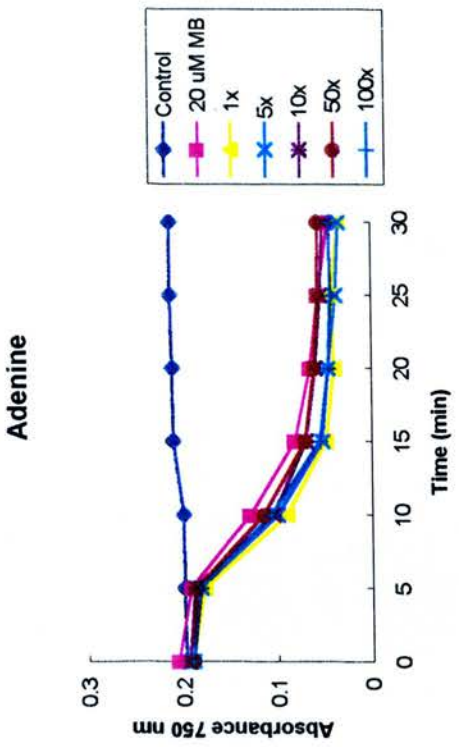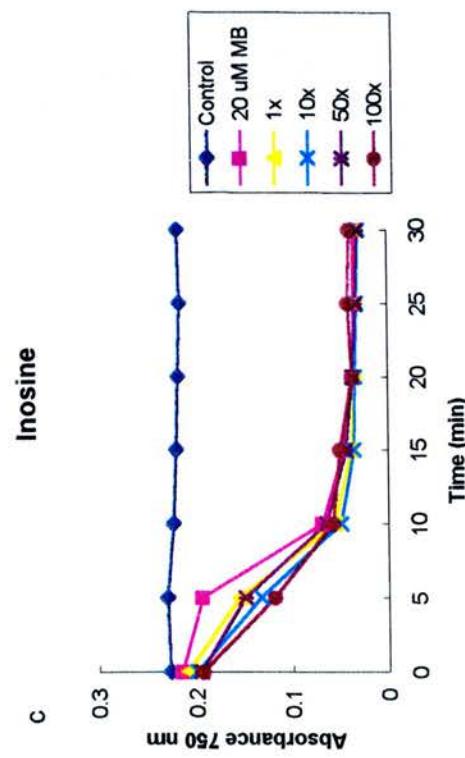### 5.3.7.3. Inhibition of mel B lysis by Berenil®

Berenil® inhibited lysis by 20 μM mel B in the culture-adapted trypanosomes, in a dose-dependent manner (Figure 5.8d). Approximately 90% protection was achieved with 50x excess concentrations of Berenil®. At 100x excess there was complete inhibition of lysis. Intermediate inhibitions were observed at 10x excess Berenil®. 1x excess Berenil® did not inhibit lysis.

5.3.8. Stability of mel Cy- induced drug resistance using *in vitro* lysis assay

5.3.8.1 Lysis of drug resistant trypanosomes growing in the presence of drug

Lysis of the intermediate stages of drug resistant trypanosomes growing in the presence of the maximum selective dose of mel Cy in mice is shown in Figures 5.9a and b. The CR$_{9d}$ trypanosomes passaged 9 times in the presence of drug were not lysed by 10 μM mel Cy (Figure 5.9a). The CR$_{11d}$ passaged 11 times, while still in the presence of drug, were not lysed by 20 μM mel Cy. At concentrations of 50-100 μM mel Cy lysis was delayed for 25 minutes. Rapid lysis of these parasites occurred at 250 μM mel Cy. This shows a high increase in tolerance to mel Cy by trypanosomes maintained in the presence of drug.
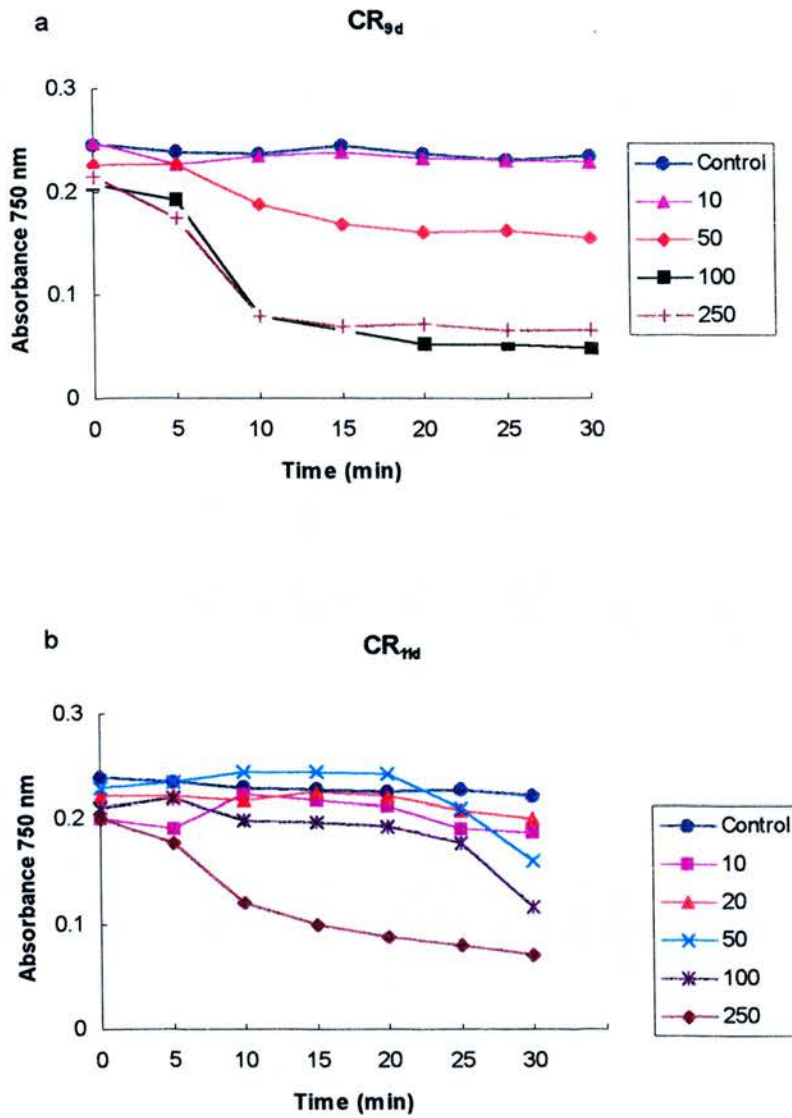
Figure 5.9: Lysis of *in vivo*-derived drug resistant *Trypanosoma evansi* growing in the presence of selective concentrations of mel Cy. Trypanosomes selected with 40 mg/kg mel Cy were passaged, a, 9 times (CR$_{9d}$); and, b, 11 times (CR$_{11d}$) in the presence of 40 mg/kg mel Cy. Lysis assays were carried out before withdrawal of drug, using increasing concentrations of mel Cy. Dose of mel Cy are in μM concentrations of mel Cy.

5.3.8.2. *Lysis of in vitro-derived drug resistant Trypanosoma evansi growing in the absence of drug by mel Cy.*

In vitro-derived resistant trypanosomes growing in the absence of drug for up to 4 weeks were tested for lysis by mel Cy at different time points after withdrawal of drug. The results are shown in Figures 5.10a-c. At 12, 19 and 26 days following withdrawal of drug trypanosomes were not lysed by 50 µM mel Cy. Little lysis occurred at 100 µM and rapid lysis was only observed at 250-500 µM mel Cy. These observations were similar to those on the main stabilate before drug was withdrawn for the stipulated periods of time (see Figure 5.2b).

A summary of experiments and the characteristics of the different *T. evansi* lines using *in vitro* lysis assay is given in Table 5.1.
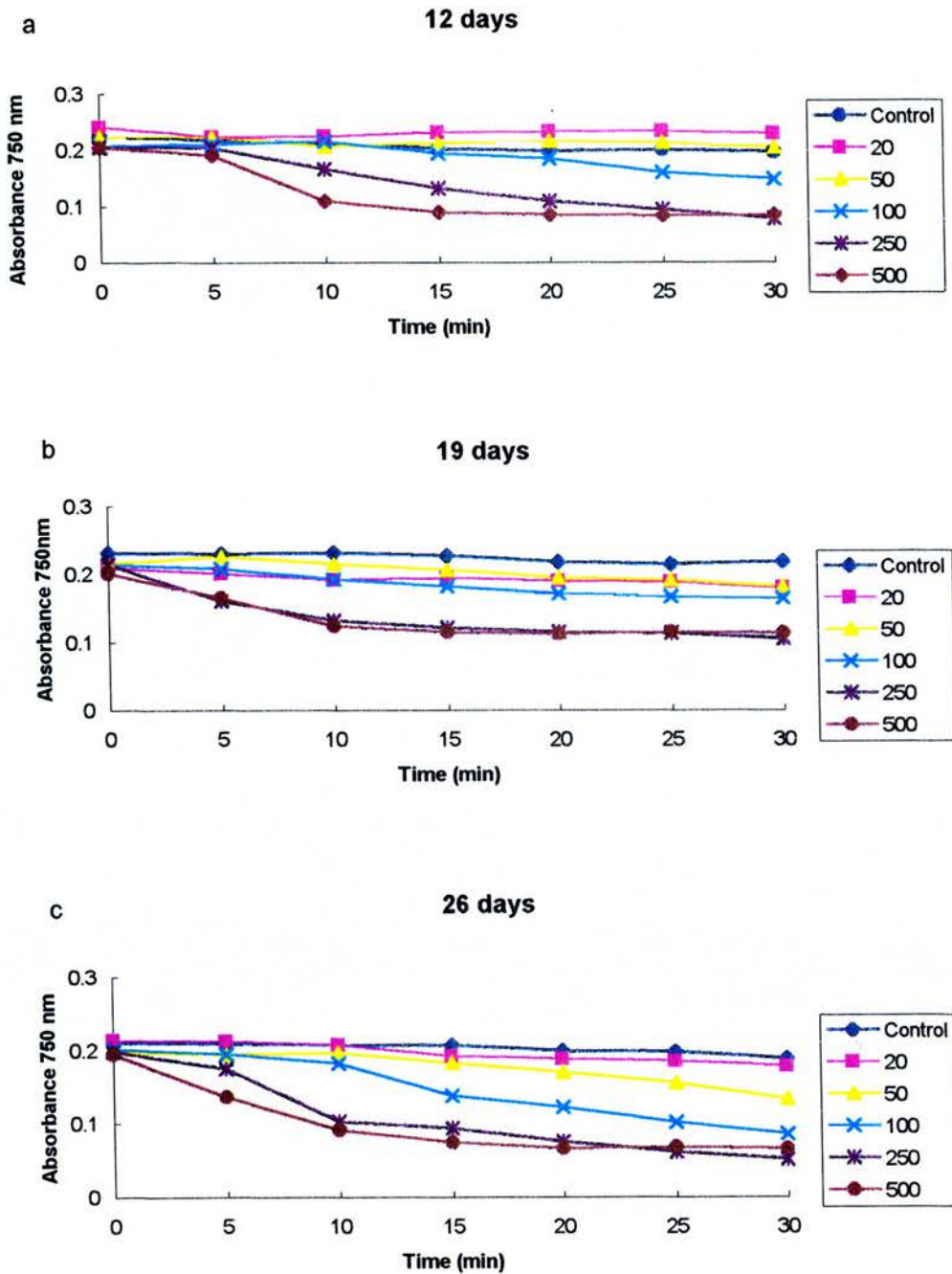
Figure 5.10: Lysis of *in vitro*-derived drug resistant *Trypanosoma evansi* (MCR1.1) by mel Cy after cultivation in the absence of drug for a, 12 days, b, 19 days and, c, 26 days. After completion of induction *in vitro* drug was withdrawn from resistant cultures and lysis assays using mel Cy were carried out at the specified number of days. Lysis assays were carried out as described above. No reductions in resistance were observed. Doses of mel Cy are in μM.

Table 5.1: Summary of characterisations of drug sensitive and drug resistant *T. evansi* lines using *in vitro* lysis assay.

T. evansi stock	Lysis							Inhibition of lysis							
	Mel Cy (µM)			Mel B (µM)				1 µM Mel Cy				20 µM Mel B			
	1	10-50	100	10	20	50	100	Ads	Adn	Ino	BN	Ads	Adn	Ino	BN
WT	+++			±	++	+++	+++	×××	×××	-	×××	-	-	-	×××
CR2	-	-	±												
CR3	-	±	+												
CR3.1	-	±	+	-	-	++	+++								
CR$_{9d}$	-	+	+++												
CR$_{11d}$	-	±	±												
WTAX	+++			+	+++			×××				-	-	-	×××
MCR1.1	-	-	+	-	-	-	±								
MCR1.1st	-	±	±												

- = No detectable lysis; ± = partial lysis; + = little lysis; ++ = moderate, incomplete lysis and +++ = rapid lysis
××× = inhibition.

5.4 DISCUSSION

To investigate the mode of uptake of melaminophenyl arsenical drugs into *T. evansi* the susceptibility of these parasites to *in vitro* lysis by mel Cy and mel B was studied. Competition for uptake on the same transporter by different purine compounds, as well as diamidines was also investigated.

The results show that mel Cy and mel B caused lysis of drug sensitive and drug resistant *T. evansi* to different extents. The partial lysis of drug resistant trypanosomes at low concentrations of drug observed in this study has been previously reported: Scott *et al.* (1997) measured lysis of mel Cy-selected *T. brucei* by 4 different arsenical drugs, mel Cy, mel Ox (melarsen oxide), mel B and phenyl arsenoxide. The authors similarly found that arsenical resistance was expressed to all the four arsenicals to different extents. Concentrations of mel Ox up to 100 µM had no noticeable effect on the mel Cy resistant isolate (247MelCy^R). However, mel Cy caused partial lysis at 50 µM concentration, similar to our findings. More lysis occurred at 500-1000 µM mel Cy, but lysis was neither rapid nor complete at these high concentrations, similar to results presented here.

Other authors have reported higher doses for lysis of drug resistant trypanosomes by arsenicals. Yarlett *et al.* (1991) found that arsenical resistant *T. rhodesiense* isolates required up to 37-fold higher concentrations of melarsen oxide to cause lysis within 30 minutes. The trypanosome strain isolated from patients who relapsed after several courses of mel B treatment required at least 18-fold greater concentrations of melarsen oxide to cause lysis, compared to the susceptible strains. Fairlamb *et al.* (1992b) found that melarsen-resistant *T. brucei* was completely unaffected by melarsen oxide concentrations up to 100 µM, indicating a 200-fold resistance compared to the drug sensitive population.

The pattern of lysis by mel Cy in the drug resistant trypanosomes derived in this study indicates that these parasites still maintained some level of sensitivity to mel Cy *in vitro*. The differences between the resistant and sensitive populations only lay in the concentration and time of exposure to drug. This may suggest that the resistant

trypanosomes concentrate the arsenical but at a slower rate compared to the drug sensitive parasites.

The *in vitro* -derived drug resistant line (MCR1.1) showed higher resistance to lysis by mel Cy compared to the mouse-derived resistant lines. The *in vitro* system may thus represent a more efficient method of selection of drug resistant trypanosomes. It was also observed (see Chapter 4) that the *in vitro*-derived resistance was more stable in the absence of drug than the *in vivo*-derived resistance. The differences in susceptibility to *in vitro* lysis between these resistant lines may be a result of the differences in stability of resistance in the two populations.

The inhibition of mel Cy lysis by adenosine and adenine indicate that these compounds compete with mel Cy for the same transporter, suggesting a common mode of uptake. This is in agreement with the findings of Carter and Fairlamb (1993) on *T. brucei* using melarsen oxide. Similar inhibitions of arsenical lysis by adenine and adenosine were reported by Scott *et al.* (1997), and Ross and Barns (1996).

The dose dependent and saturable inhibition of mel Cy lysis by Berenil® means that inhibition was competitive. This suggests that Berenil® and mel Cy interact with a common transporter. This agrees with previous reports on melaminophenyl arsenical drugs and the diamidines (Carter and Fairlamb, 1993; Scott *et al.*, 1997; Ross and Barns, 1996).

The existence of a common transport mechanism has been suggested as an explanation for cross resistance between melaminophenyl arsenicals and diamidines in *T. brucei* (Carter and Fairlamb, 1993) and *T. equiperdum* (Barrett *et al.*, 1995). The benzamidine rings in the diamidines and the melamine ring in the melaminophenyl arsenicals have been proposed as similar structures that are recognised by the transporter (Fairlamb *et al.*, 1992b).

That transport of melaminophenyl arsenicals takes place by a specific selective mechanism was demonstrated using a lipid soluble trivalent arsenical, phenyl arsenoxide, which lacks the melamine ring (Carter and Fairlamb, 1993). Lysis of *T. brucei* by phenyl arsenoxide was not inhibited by 100x excess concentrations of

adenine, adenosine or dipyridamole, *in vitro*, suggesting that this compound did not enter trypanosomes via purine transport system. It is thought that due to its lipid solubility phenyl arsenoxide diffuses passively across the lipid membrane of trypanosomes without requiring a specific transporter. This may explain why it is not inhibited by purine transport inhibitors.

The uptake of another melaminophenyl arsenical, mel B was also investigated in this study. It was observed that mel B was less effective in causing lysis of the drug sensitive trypanosomes. On the other hand it was very potent in lysing drug resistant trypanosomes. Similar observations on mel B lysis have been reported by Yarlett *et al.* (1991) and Scott *et al.* (1997). Yarlett, *et al.* (1991) found that in susceptible isolates of *T. rhodesiense* amount of drug causing 50% lysis (L_{50}) was higher for mel B compared to melarsen oxide. In contrast, in arsenical resistant isolates lower L_{50} were consistently observed for mel B over melarsen oxide. These resistant parasites required 17-37 fold higher concentrations of melarsen oxide but 3-10 fold concentrations of mel B to cause lysis within 30 minutes. Scott *et al.* (1997) also found that approximately 25-fold higher concentrations of mel B was required to bring about lysis similar to that observed at 1 μM mel Cy in drug sensitive *T. brucei*. On the contrary mel B was the most effective of three melaminophenyl arsenicals at lysing drug resistant parasites, with partial lysis occurring at 50 μM, similar to our results.

This study showed that mel Cy is a more potent trypanocide *in vitro* compared to mel B.

Absence of competitive inhibition of mel B lysis by adenosine, adenine and inosine suggests that mel B, though a melaminophenyl arsenical, may not be transported into trypanosomes via an adenosine transport system.

It is interesting that Berenil® inhibited lysis by both mel Cy and mel B, since lysis by mel Cy was inhibited by adenine and adenosine and lysis by mel B was not. It means there is a common transport system between Berenil® (diamidine) and the two melaminophenyl arsenicals. If this is the case then it also means that melaminophenyl arsenicals have more than one mechanisms of uptake into drug

sensitive trypanosomes. One of these must be an adenosine transport system which mediates the transport of mel Cy and other melaminophenyl arsenicals such as melarsen oxide, but not mel B. Berenil® transport into trypanosomes may also involve more than one transporter: an adenosine transporter shared with mel Cy, and an alternative unidentified transport mechanism shared with mel B. Alternatively it may be associated with a non- adenosine transport system which is shared by both mel Cy and mel B.

Scott *et al.* (1997) also found that mel B induced lysis was not blocked by adenine and adenosine but it was blocked by Berenil® and pentamidine. These authors found that Berenil® and pentamidine inhibited lysis by two other melaminophenyl arsenicals, mel Ox and mel Cy. He also considered the possibility of a non-adenosine transporter for these compounds.

The results presented here argue against the suggestion of one transporter for the uptake of all melaminophenyl arsenicals. It is possible that there are some unidentified alternative routes of uptake of some melaminophenyl arsenicals such as mel B into trypanosomes. It is possible that the lipid soluble mel B diffuses passively into the trypanosome as an alternative pathway. However the fact that lysis by mel B was inhibited in a clear dose dependent manner by Berenil® but not by adenine or adenosine, show that a more specific selective mechanism of uptake is involved.

This study suggests that an alternative non-adenosine transporter which has not yet been identified may mediate for uptake of some melaminophenyl arsenicals into trypanosomes, in addition to the P2 adenosine transporter.

Based on concentration of arsenical required to cause lysis it was possible to differentiate the drug sensitive from the drug resistant trypanosomes using the *in vitro* lysis assay. This assay was also able to demonstrate the cross resistance between mel Cy and mel B detected using *in vitro* growth inhibition assays. The assay further confirmed the differences in stability of resistance between drug resistant lines developed in *vivo* and in *vitro.* These all show that the simple lysis assay is a suitable test for identification of arsenical resistant trypanosome.

The observations on the lysis of trypanosomes by both mel Cy and mel B and the inhibitions of lysis by the two arsenicals was broadly similar in both the *in vivo*-derived and the *in vitro*-derived trypanosome lines. This again shows that *in vitro* systems can be conveniently used as substitute for animal models in studies of mechanisms of drug resistance.

CHAPTER SIX

STUDIES ON ADENOSINE UPTAKE IN DRUG SENSITIVE AND DRUG RESISTANT *TRYPANOSOMA EVANSI*

6.1. INTRODUCTION

Purine and pyrimidine nucleotides function as precursors of nucleic acid (DNA and RNA synthesis), carriers of high-energy phosphate bonds, modulators of enzyme activities and constituents of certain co-enzymes, besides their involvement in sugar and energy transfer reactions (Hammond and Gutteridge, 1984). Many mammalian cells possess two pathways for the synthesis of purine nucleotides. The most common is the *de novo* pathway. This produces inosine monophosphate (IMP) from simple precursors such as glycine and formate. Some other mammalian cells rely on salvage of preformed purine bases and nucleosides present in the circulatory system (Hammond and Gutteridge, 1984).

Studies on several parasites, e.g. *Trichomonas foetus, T. vaginalis,* (Wang, *et al.,* 1983), *Plasmodium* species (Hensen *et al.,* 1980), *Trypanosoma brucei, T. congolense* (James and Born, 1980), *T. rhodesiense* (Sanchez *et al.,* 1976), and *Leishmania* promastigotes (Aronow *et al* , 1987; Ogbunude *et al.,* 1991; Baer *et al.,* 1992) show that protozoans in general are unable to synthesise their own purines (*de novo* synthesis). They therefore rely on salvage pathways to utilise purines from the host fluids.

The purine ring is commonly found and available to the parasite as nucleosides, nucleobases, as charged nucleotides, or as polymers in nucleic acids. Investigations on the acquisition and metabolism of the purine ring by trypanosomatids have demonstrated that these pathways are distinct from those used by mammals (Hammond and Gutteridge, 1984; Cohn and Gottlieb, 1997). Presently, knowledge of the biochemical pathways as well as enzymes involved in purine metabolism in

trypanosomatids is leading to the development of trypanocidal drugs based on substrate analogs (Craig and Eakin, 1997).

The acquisition of the purine ring begins with the transport of purine nucleosides and nucleobases across the plasma membrane. Besides being permeable to these substrates purines and purine derivatives are absorbed into parasites by specific mediated transport processes (Henson *et al.*, 1980; Aronow *et al.*, 1987). Transport of purines across parasites's plasma membrane also presents a promising target for the control of diseases caused by members of Trypanosomatidae (Cohn and Gottlieb, 1997).

To date no purine transporter *per se* has been isolated from any trpanosomatid (Cohn and Gottlieb, 1997). However, studies on transport of nucleosides in several species of protozoa (Manjra and Dusanic, 1973; Kidder *et al.*, 1978; James and Born, 1980; Henson *et al.*, 1980; Wang *et al.*, 1983; Ogbunude *et al.*, 1991; Baer *et al*, 1992), suggest that there is considerable variation in the expression of purine transport systems in these parasites. *L. donovani*, for example was shown to possess two transporters with preferential ability to transport adenosine and inosine (Aronow *et al.*, 1987). Similar studies on *T. brucei* and *T. congolense* (James and Born, 1980) showed that adenosine was the most important purine for these parasites.

Adenosine transport has recently been implicated in the mechanism of resistance to arsenical drugs (Carter and Fairlamb, 1993). This study showed that melarsen-sensitive *T. brucei* have two high affinity adenosine transport systems. A P1 type which also transports inosine, and a P2 type which also transports adenine and melaminophenyl arsenicals. A melarsen-resistant clone of *T. brucei* was found to have lacked P2 transport suggesting that resistance to these arsenicals was due to loss of uptake. Investigations on the mode of uptake of arsenical drugs using mel Cy and mel B in this study (see Chapter 5) indicate that melaminophenyl arsenicals may have more than one mechanism of uptake into trypanosomes.

The study presented in this chapter was an attempt to further explore into the mechanisms of adenosine uptake in drug sensitive *T. evans*i, using adenosine uptake

inhibition. The study also evaluates how these transport functions are altered in drug resistant populations. The specific aims include the following:

1. To investigate the specific uptake pathways by which adenosine is taken up into *T. evansi* in comparison with other related species of trypanosomes.

2. To assess the extent to which alternative substrates affect the uptake of adenosine on these transporters.

3. To investigate the basis of cross resistance between diamidines and melaminophenyl arsenicals observed in this and other reported studies.

6.2 . MATERIALS AND METHODS

6.2.1. Trypanosomes

Studies were carried out using trypanosomes maintained in mice (WT, CR3, CR3.1) as well as those cultivated *in vitro* (WTAX, MCR and MCR1.1).

Cryopreserved blood stabilates of mice-maintained trypanosome lines were grown in two mice for use in each experiment. Trypanosomes at high parasitaemia (approximately 4×10^8/ml) were separated from mice blood by anion exchange chromatography, as described in the General Methods. *In vitro*-maintained trypanosomes were harvested from healthy growing cultures in logarithmic phase of growth.

Trypanosomes obtained from either mice isolations or culture suspensions were washed in PSG (pH 8.0) by centrifugation at 1000x g for 10 minutes. The pellet was resuspended twice in 1.5 ml of PSG in sterile Eppendorf tube, and centrifuged at 100x g for 6 minutes each time, using a biofuge. The trypanosomes were finally resuspended in 1 ml of uptake ɔuffer (CBSS, pH 7.4, Fairlamb, *et al.*, 1992a) containing 1% bovine serum albumin (BSA). A 1:50 dilution of the trypanosome suspension was counted using a Neubauer haemocytometer, and density of cells adjusted to 1.25×10^8/ml using uptake buffer.

6.2.2. Uptake assays

Tritiated adenosine was prepared by diluting 5 µl to each 1 ml of 5 µM nonradiolabelled (cold) adenosine. Aliquots of 80 µl of prewarmed cells containing 10^7 trypanosomes in uptake buffer were added to each incubation tube. Inhibitors consisting of adenine, inosine, deoxyadenosine or Berenil® were prepared at 50x the required concentration in assay, and 2 µl volumes were added to incubation mixture. Uptake was initiated by the addition of 5 µM tritiated adenosine (final concentration in assay was 1 µM; specific activity 2.5 mCi mmol^{-1}) to each incubation tube at 20 seconds intervals. Reaction mixtures were incubated for 5 minutes at 37°C.

At the end of incubation 100 µl of ice cold 1 mM nonradiolabelled adenosine solution in PBS (Stop Solution) was added to incubation medium. The whole mixture was transferred quickly to an Eppendorf filtration unit containing 0.45 µm cellulose acetate filter (COSTAR). Filtration tubes were centrifuged at maximum speed (100x g) for 40 seconds in a biofuge. The filters were rinsed with 0.5 ml of Stop Solution and centrifuged at 100x g for 1 minute. The top of the Eppendorf tube was snipped off using a pair of scissors. The filter holder was removed and the top part snipped off using a "micro tube cutter". The lower piece containing the filter membrane was transferred to a scintillation vial and dried in an oven at 50°C, overnight (or at 100°C for 1.5 hours). One ml of scintillation fluid (PACKARD, UK) was added to each scintillation vial, the cap screwed tight and the amount of radioactivity incorporated read in a scintillation counter.

The amount of adenosine uptake was calculated from values of incorporated radioactivity (counts per minute, CPM) and expressed as pmoles/10^8 trypanosomes.

Control incubations consisting of trypanosomes and tritiated adenosine with no inhibitor added were included in every experiment. Also blanks consisting of uptake buffer and Tritiated adenosine with no trypanosomes were included to estimate for non-specific binding of radioactivity. Duplicate tubes were used for each assay. Experiments were repeated 2-4 times. The average of the readings of the blank incubations were deducted from each experimental data.

The amount of adenosine uptake in control incubations consisting of trypanosomes in the absence of inhibitor represented total (100%) uptake. The amount of inhibition of adenosine uptake in the presence of each inhibitor was calculated as percentage of control.

The effects of inhibition of adenosine uptake by 1 mM adenine, 1 mM inosine or 100 µM Berenil® were measured as single inhibitors or in combination with one or the other inhibitor, in both the drug sensitive and drug resistant populations. The effect of increasing concentrations of the different inhibitors on uptake of 1 µM adenosine in the presence of either adenine or inosine were also measured. Concentrations ranged from 0.01-1000 µM. Both drug sensitive and drug resistant trypanosomes were characterised.

6.3. RESULTS

6.3.1. Inhibition of adenosine uptake in *Trypanosoma evansi* maintained in mice

6.3.1.1. Drug sensitive line

The results of inhibition of adenosine uptake in the mouse-maintained unselected *T. evansi* clone (WT) using 1 mM adenine, 1 mM inosine and 100 µM Berenil®, either singly or in combination are shown in Figure 6.1.

Inosine, adenine and Berenil® all inhibited uptake of adenosine to variable extents. Inosine caused 23.2± 2.9% inhibition of total uptake at 1 mM concentration, giving a residual uptake of 76.8%. One mM concentration of adenine caused 53.6±3.8% inhibition, giving a residual uptake of 46.4%. Taken together both inosine and adenine inhibited a total of 76.9% (range 66.1-100.4%) of total adenosine uptake in these parasites. However in the presence of a combination of both 1 mM adenine and 1 mM inosine uptake of 1 µM adenosine was completely inhibited (99.0±0.3%).

also mediating the transport of inosine and the other adenine, similar to *T. brucei* (Carter and Fairlamb, 1993).

Deoxyadenosine, a deoxidised form of adenosine is similar in structure to adenosine except for the absence of one oxygen atom in the pentose sugar unit of the molecule, caused 97.1±0.6% inhibition of adenosine uptake.

Berenil[®] at 100 µM concentration inhibited 33.2±2.3% of adenosine uptake. In the presence of adenine addition of 100 µM Berenil[®] did not result to further increase (50.5 ±6.1%) in inhibition of adenosine uptake beyond that caused by adenine alone (53.6±3.8%). On the other hand, addition of 100 µM Berenil[®] in the presence of 1 mM inosine resulted to a 4-fold increase in inhibition (88.4±2.6%) of adenosine uptake beyond that caused by inosine alone (23.2± 2.9%).

WT
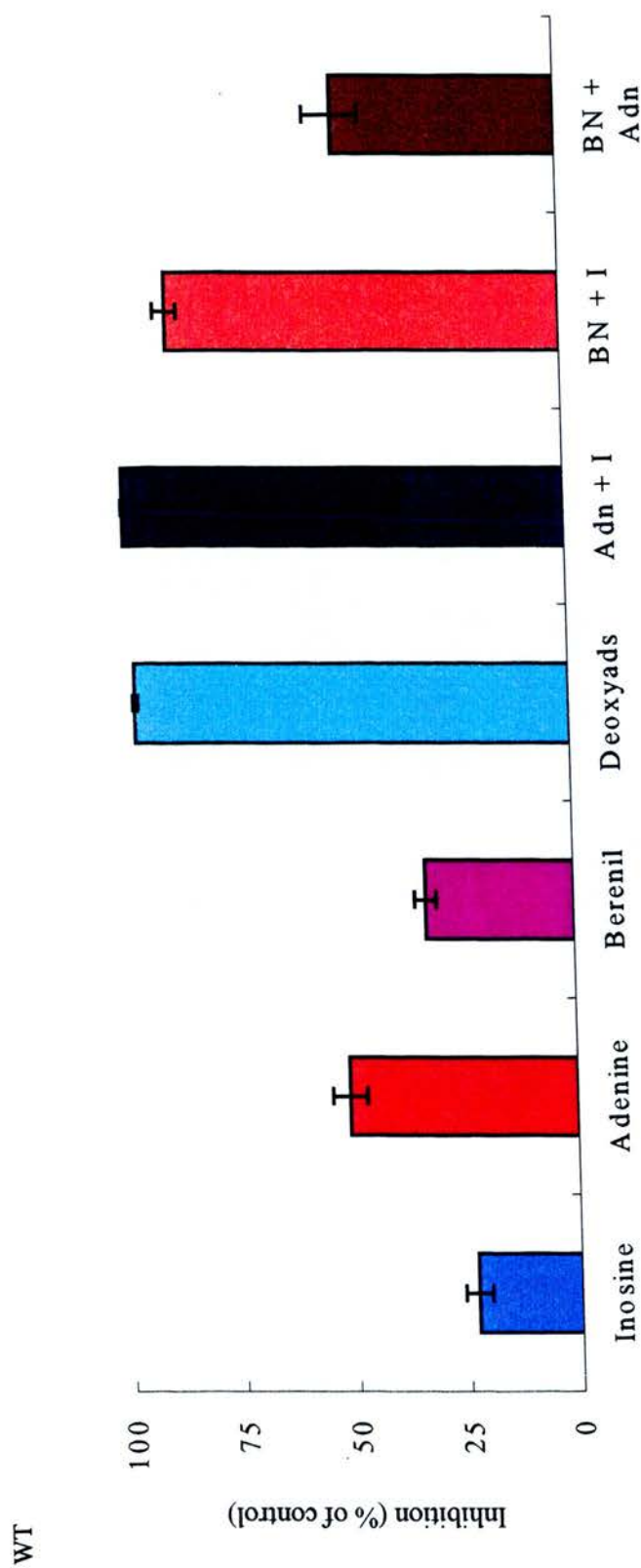
Figure 6.1: Inhibition of uptake of 1 μM adenosine in the *in vivo*-maintained drug sensitive *Trypanosoma evansi* (WT). 1x10^{7} trypanosomes in 80 μl of uptake buffer were incubated with 1 μM tritiated adenosine at 37°C, for 5 minutes. Inhibitors consisting of 1 mM inosine (I), 1 mM adenine (Adn), 1 mM deoxyadenosine (Deoxyads) or 100 μM Berenil® (BN); either singly or in combination were added in 2-4 μl volumes. Reaction mixtures were filtered by centrifugation through 0.45 μm acetate filter (COSTAR), at 100x g. Trypanosomes trapped on filters were washed with 0.5 ml of non radiolabelled (cold) adenosine. Filters were dried at 50°C overnight. Incorporated radioactivity was read in a scintillation counter. Uptake of adenosine was calculated from counts of decompositions of radioactivity. Amount of inhibition was calculated as percentage of uptake of control incubations in the absence of inhibitor subtracted from 100. Bars represent standard errors of means of at least 4 experiments.

6.3.1.2. Drug resistant line

Figure 6.2 shows results of inhibition of adenosine uptake in the *in vivo*-derived drug resistant population, CR3. In these trypanosomes, adenine, inosine and Berenil® (as well as deoxyadenosine) also caused varying levels of inhibition of uptake of adenosine. However there were marked changes in the activity of the individual transporters when compared with the wild type. Inosine alone caused 81.0±2.8% inhibition at 1 mM concentration. Hence uptake at the alternate transporter was approximately 19.0%. Adenine alone inhibited 38.1±1.3% of uptake leaving a residual uptake of approximately 61.9%. This represents a 4-fold decrease in uptake at the adenine/adenosine (P2) transporter and 1.3-fold increase in the inosine/adenosine (P1) transporter (Carter and Fairlamb, 1993).

In the drug resistant parasites a combination of 1 mM adenine and 1 mM inosine also led to complete (98.9±0.5%) inhibition of uptake of 1 μM adenosine suggesting that two adenosine transporters were present in the drug resistant population. One is inhibited by inosine and the other by adenine.

Inhibition by 1 mM deoxyadenosine was also similar to that observed in the unselected line with percentage inhibition of 96.1±0.6

Hundred μM Berenil® alone inhibited 30.7±4.5% of adenosine uptake. In combination with inosine there was almost 4-fold increase in inhibition by Berenil® (98.5±1.4%), similar to the observations on the drug sensitive line. However complete inhibition was not achieved with Berenil® in the presence of inosine.

Interestingly, combination of Berenil® with 1 mM adenine in these drug resistant parasites resulted to a 2-fold increase in inhibition (70.0±9.9) beyond that caused by adenine alone (38.1±1.3%). This increased inhibition was not observed in the drug sensitive clone.

Results of inhibition of adenosine uptake in the second *in vivo*-derived drug resistant line, CR3.1 is shown in Figure 6.3. Inhibitions were similar to those observed on the CR3 parasites. Inosine inhibited 66.3±0.8%, giving a residual uptake at the
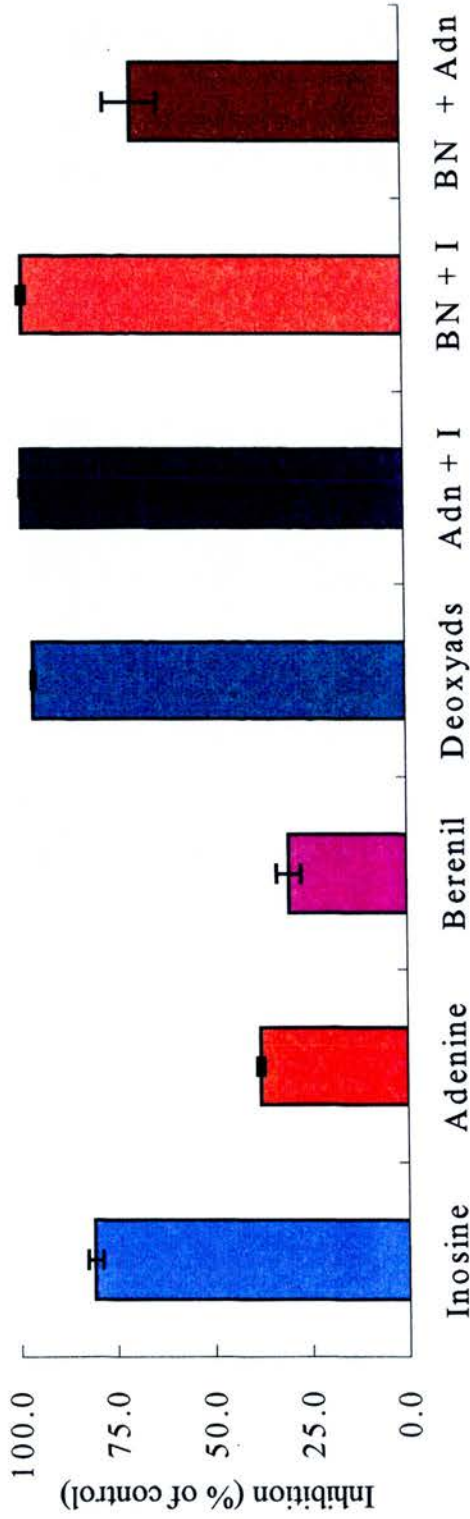
Figure 6.2: Inhibition of uptake of 1 μM adenosine in the *in vivo*-derived drug resistant *Trypanosoma evansi* (CR3). 1×10^7 trypanosomes in 80 μl of uptake buffer were incubated with 1 μM tritiated adenosine at 37°C, for 5 minutes. Inhibitors consisting of 1 mM inosine (I), 1 mM adenine (Adn), 1 mM deoxyadenosine (Deoxyads) or 100 μM Berenil® (BN); either singly or in combination were added in 2-4 μl volumes. Reaction mixtures were filtered by centrifugation through 0.45 μm acetate filter (COSTAR), at 100x g. Trypanosomes trapped on filters were washed with 0.5 ml of non radiolabelled (cold) adenosine. Filters were dried at 50°C overnight. Incorporated radioactivity was read in a scintillation counter. Uptake of adenosine was calculated from counts of decompositions of radioactivity. Amount of inhibition was calculated as percentage of uptake of control incubations in the absence of inhibitor subtracted from 100. Bars represent standard deviations of means of 2 experiments.

CR3.1

Figure 6.3: Inhibition of uptake of 1 µM adenosine in the *in vivo*-derived drug resistant *Trypanosoma evansi* (CR3.1). Inhibitors 1x10⁷ trypanosomes in 80 µl of uptake buffer were incubated with 1 µM tritiated adenosine at 37°C, for 5 minutes. Inhibitors consisting of 1 mM inosine (I), 1 mM adenine (Adn), or 100 µM Berenil® (BN); either singly or in combination were added in 2-4 µl volumes. Reaction mixtures were filtered by centrifugation through 0.45 µm acetate filter (COSTAR), at 100x g. Trypanosomes trapped on filters were washed with 0.5 ml of non radiolabelled (cold) adenosine. Filters were dried at 50°C overnight. Incorporated radioactivity was read in a scintillation counter. Uptake of adenosine was calculated from counts of decompositions of radioactivity. Amount of inhibition was calculated as percentage of uptake of control incubations in the absence of inhibitor subtracted from 100. Duplicate experiments were not carried out with this line.

adenine/adenosine transporter of 33.8%. This represents a 2.7-fold decrease in uptake compared to the unselected (WT) population. Adenine inhibited 35.4±16.8 giving a residual P1 uptake of 64.6% which represents a 1.4-fold increase in uptake at this transporter. Again complete inhibition (100.0%) was observed in the presence of both adenine and inosine, suggesting the presence of a bipartite adenosine transport system in these parasites.

In this drug resistant clone Berenil® alone inhibited 17.3% of adenosine uptake, which represents a 2-fold decrease in the amount of inhibition by Berenil®. Combination of Berenil® and inosine resulted to 96.4% inhibition of uptake similar to the CR3. Addition of Berenil® in the presence of adenine resulted to 65.3% inhibition, representing 1.5-fold increased inhibition beyond that produced by adenine alone.

6.3.2. Inhibition of adenosine uptake in the culture-adapted *Trypanosoma evansi* lines

6.3.2.1. Drug sensitive trypanosomes

Results of inhibition of adenosine uptake in the culture-adapted unselected *T. evansi* (WTAX) is shown in Figure 6.4. Inosine inhibited 20.5±3.7% of total adenosine uptake, giving a residual P2 uptake of 79.5%. Adenine inhibited 82.2±11.7% of total adenosine uptake, giving a residual P1 uptake of 17.8%. Similar to the WT parasites the presence of saturating concentrations of both inosine and adenine led to complete inhibition of adenosine uptake in the WTAX parasites.

Deoxyadenosine caused inhibition of 89.5±0.8% in these parasites.

Similar observations to those on the mouse-maintained WT were made on inhibitions by Berenil® in the WTAX. Berenil® inhibited 42.4±5.4% of total adenosine uptake. A combination of 100 μM Berenil® and 1 μM adenine did not result to further increase in inhibition (80.2±6.1%) to that caused by adenine (82.2±11.7%). Again addition of 100 μM Berenil® in the presence of 1 mM inosine resulted to 3.9-fold increase (80.7±11.9%) in inhibition caused by inosine alone (20.5±3.7%).

WTAX
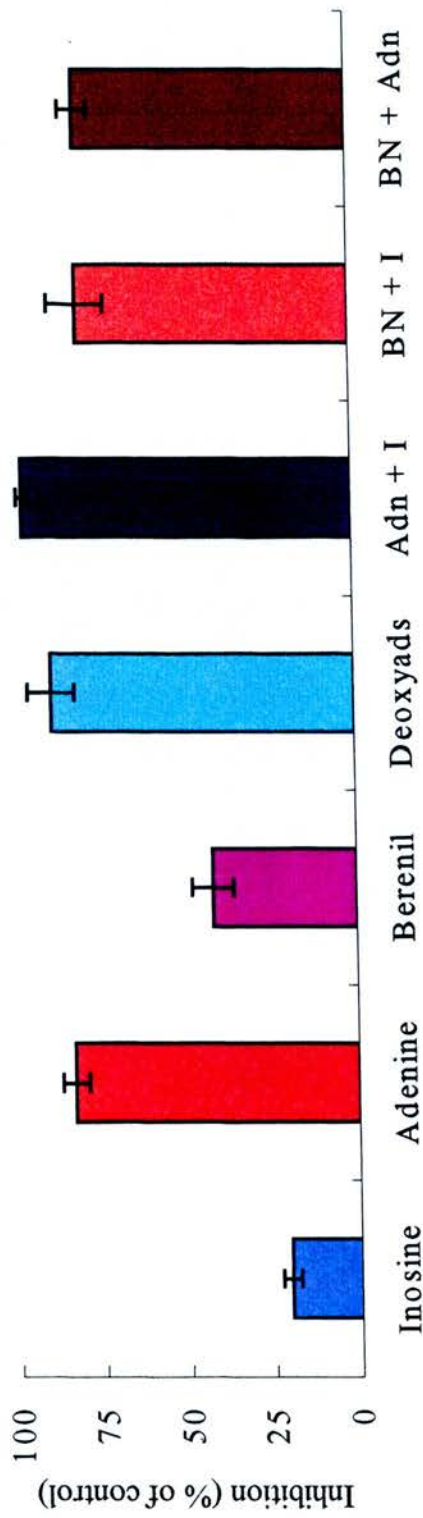
Figure 6.4: Inhibition of uptake of 1 μM adenosine in the culture-adapted drug sensitive *Trypanosoma evansi* (WTAX). 1x10⁷ trypanosomes in 80 μl of uptake buffer were incubated with 1 μM tritiated adenosine at 37°C, for 5 minutes. Inhibitors consisting of 1 mM inosine (I), 1 mM adenine (Adn), 1 mM deoxyadenosine (Deoxyads), or 100 μM Berenil® (BN); either singly or in combination were added in 2-4 μl volumes. Reaction mixtures were filtered by centrifugation through 0.45 μm acetate filter (COSTAR), at 100x g. Trypanosomes trapped on filters were washed with 0.5 ml of non radiolabelled (cold) adenosine. Filters were dried at 50°C overnight. Incorporated radioactivity was read in a scintillation counter. Uptake of adenosine was calculated from counts of decompositions of radioactivity. Amount of inhibition was calculated as percentage of uptake of control incubations in the absence of inhibitor subtracted from 100. Bars represent standard errors of means of at least 3 experiments.

6.3.2.2. Drug resistant lines

Figure 6.5 shows results of inhibition of uptake of 1 μM adenosine in the *in vitro*-derived drug resistant population, MCR1. Similar to the *in vivo*-derived lines there were marked changes in activity of adenosine transporters in these resistant trypanosomes. Inosine alone inhibited 92.4±5.6 % of total adenosine uptake as opposed to 20.5% in the wild type, giving a residual P2 transport of 7.6%. This represented over 10-fold decrease in P2 uptake compared to the drug sensitive (WTAX) parasites. Adenine on the other hand inhibited 44.6±9.9% of adenosine uptake giving a P1 transport of 55.4%. This represents a 3.1-fold increase in percentage uptake at the P1 compared to the WTAX. Again almost complete inhibition (95.7±4.3%) was obtained with the addition of saturating concentrations of both adenine and inosine.

Deoxyadenosine inhibited 89.4±10.2% of total uptake.

In the MCR1 trypanosomes addition of 100 μM Berenil® in the presence of 1 mM inosine did not result to further increase in inhibition (89.6±8.6%) to that caused by inosine alone (92.4±5.6%). Again similar to observations on the *in vivo*-derived drug resistant lines (CR3 and CR3.1) addition of 100 μM Berenil® in the presence of 1 mM adenine led to a moderate (1.4 fold) increase (63.5±5.4%) beyond that caused by adenine alone (44.6±9.9).

Results of inhibition of adenosine uptake in *in vitro*-derived drug resistant clone, MCR1.1 is shown in Figure 6.6. Inhibitions were similar to those observed in the MCR1 population. Inosine inhibited 86.9±11.5% of total uptake. Hence the P2 transport was approximately 13.1%, representing a 6-fold decrease in P2 uptake. Adenine inhibited 57.4±8.3% of adenosine uptake, giving a P1 uptake of about 42.6%. This represents a 2.4-fold increase in P1 uptake compared to the drug sensitive control. Addition of saturating concentrations of both adenine and inosine resulted to complete (99.5±0.8%) inhibition of adenosine uptake in these trypanosomes.

MCR1
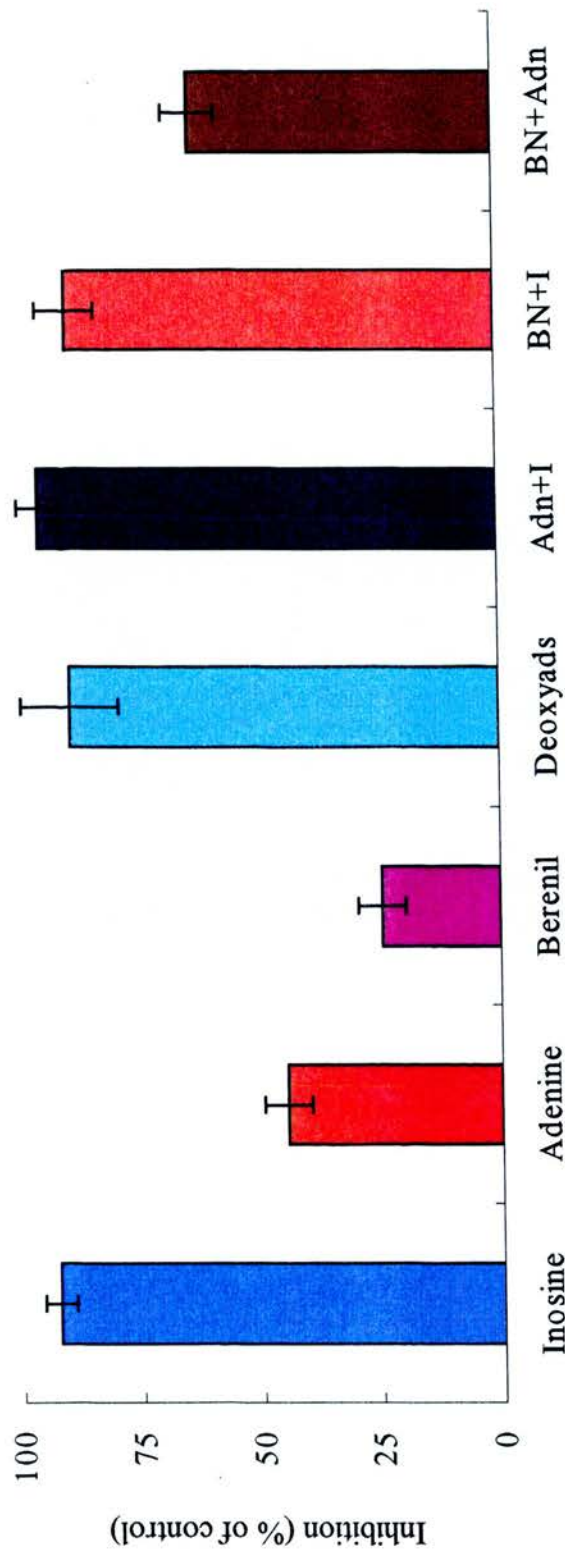
Figure 6.5: Inhibition of uptake of 1 μM adenosine in the *in vitro*-derived drug resistant *Trypanosoma evansi* (MCR1). 1x10^7 trypanosomes in 80 μl of uptake buffer were incubated with 1 μM tritiated adenosine at 37°C, for 5 minutes. Inhibitors consisting of 1 mM inosine (I), 1 mM adenine (Adn), 1 mM deoxyadenosine (Deoxyads) or 100 μM Berenil® (BN); either singly or in combination were added in 2-4 μl volumes. Reaction mixtures were filtered by centrifugation through 0.45 μm acetate filter (COSTAR), at 100x g. Trypanosomes trapped on filters were washed with 0.5 ml of non radiolabelled (cold) adenosine. Filters were dried at 50°C overnight. Incorporated radioactivity was read in a scintillation counter. Uptake of adenosine was calculated from counts of decompositions of radioactivity. Amount of inhibition was calculated as percentage of uptake of control incubations in the absence of inhibitor subtracted from 100. Bars represent standard errors of means of 4 experiments.

MCR1.1
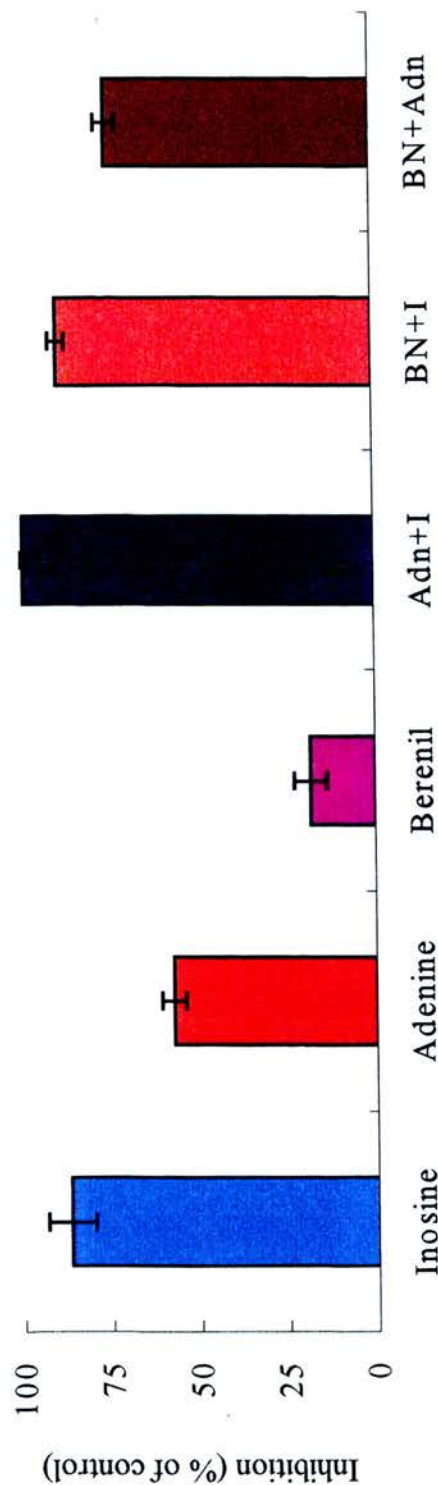
Figure 6.6: Inhibition of uptake of 1 μM adenosine in the *in vitro*-derived drug resistant *Trypanosoma evansi* (MCR1.1). 1x10^7 trypanosomes in 80 μl of uptake buffer were incubated with 1 μM tritiated adenosine at 37°C, for 5 minutes. Inhibitors consisting of 1 mM inosine (I), 1 mM adenine (Adn), or 100 μM Berenil® (BN); either singly or in combination were added in 2-4 μl volumes. Reaction mixtures were filtered by centrifugation through 0.45 μm acetate filter (COSTAR), at 100x g. Trypanosomes trapped on filters were washed with 0.5 ml of non radiolabelled (cold) adenosine. Filters were dried at 50°C overnight. Incorporated radioactivity was read in a scintillation counter. Uptake of adenosine was calculated from counts of decompositions of radioactivity. Amount of inhibition was calculated as percentage of uptake of control incubations in the absence of inhibitor subtracted from 100. Bars represent standard errors of means of 3 experiments.

Addition of 100 µM Berenil® in the presence of inosine similarly did not lead to any further increase (89.5±3.2%) in inhibition beyond that caused by inosine alone (86.9±11.5%). Similar to observations on the MCR1 population, addition of 100 µM Berenil® to 1 mM adenine resulted to a moderate (1.3-fold) increase (75.1±4.4%) in inhibition compared to inhibition by adenine alone (57.4±8.3%).

6.3.3. Effect of increasing concentrations of inhibitors on adenosine uptake

6.3.3.1. *Drug sensitive trypanosomes*

The effect of increasing concentrations of inhibitors on uptake of 1 µM adenosine was measured in the *in vivo*-maintained unselected *T. evansi* (WT), its drug resistant derivative (CR3), as well as the culture-adapted unselected line (WTAX). Results of inhibition of uptake in the WT by inosine and adenine are shown in Figure 6.7a& b. There was very little inhibition with increasing concentrations of inosine alone and a dose-dependent effect was not apparent. However, in the presence of 1 mM adenine inosine inhibited uptake in a dose-dependent manner such that at 1 mM concentration of inosine inhibition was complete. The concentration of inosine that inhibited 50% of adenosine uptake (EC_{50}) in the presence of adenine was 5.1 µM. A dose-dependent inhibition by adenine alone was more pronounced (Figure 6.7b). Inhibition increased with 1 µM adenine (22.3±7.9%) and maximum inhibition at 100 µM adenine was 56.1±9.8%. This approximates the value at 1 mM adenine (53.2%) in the single dose inhibitions shown in Figure 6.1. In the presence of 1 mM inosine higher increases in inhibition occurred with increasing concentrations of adenine. Rapid inhibition occurred at concentrations of adenine above 1 µM (48.0%) and near-saturation inhibitions were obtained at concentrations of 10-50 µM adenine (87.4-94.2%). The transporter was completely saturated at 100 µM adenine. The EC_{50} value for adenine in the presence of 1 mM inosine was 1.16 µM.

Rapid concentration dependent inhibition of adenosine uptake by adenine was also observed in the WTAX, in the presence of 1 mM inosine (Figure 6.8b), Saturation was achieved above 50 µM adenine, and the EC_{50} for this inhibition was 0.83 µM.
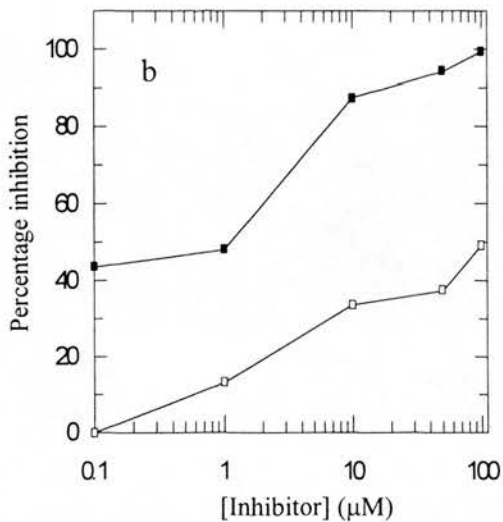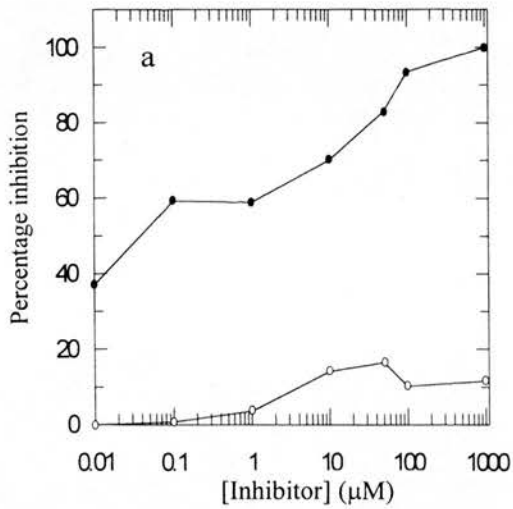
Figure 6.7 : Effect of increasing concentrations of inhibitors on uptake of 1 μM adenosine in the *in vivo*-maintained drug sensitive *Trypanosoma evansi* (WT). a, inhibition by inosine alone (open circles), and, inosine in the presence of 1 mM adenine (closed circles); b, inhibition by adenine alone (open squares) and adenine in the presence of 1 mM inosine (closed squares). 1x 10^7 trypanosomes were incubated at 37°C, for 5 minutes with 1 μM tritiated adenosine and increasing concentrations of each inhibitor. Inhibitor concentrations ranged from 0.01-1000 μM. Uptake of adenosine was measured and percentage inhibition determined, as described in the text. Each data point represents an average of at least 2 experiments.
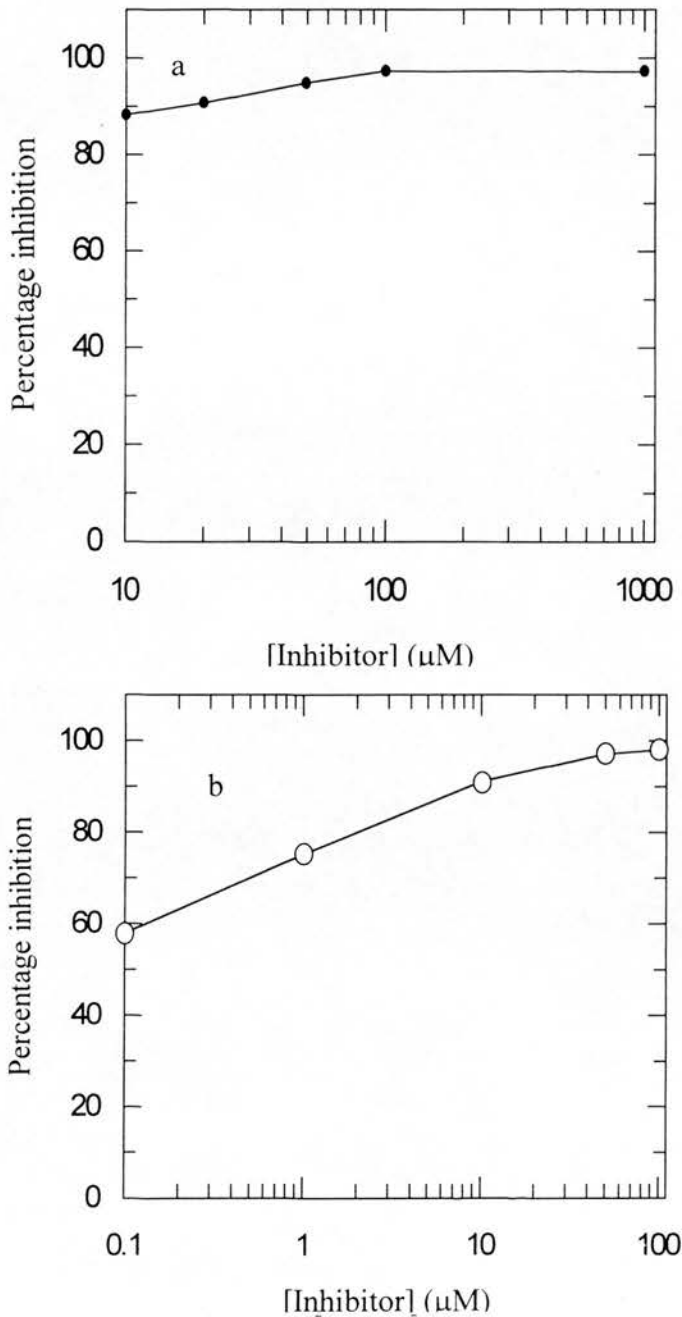
Figure 6.8: Effect of increasing concentrations of inhibitors on uptake of 1 μM adenosine in the *in vitro*-adapted drug sensitive *Trypanosoma evansi* (WTAX). a, inhibition by inosine in the presence of 1 mM adenine, and, b, inhibition by adenine in the presence of 1 mM inosine. 1x 10^7 trypanosomes were incubated at 37°C, for 5 minutes with 1 μM tritiated adenosine and increasing concentrations of either inosine or adenine. Inhibitor concentrations ranged from 0.1-1000 μM. Uptake of adenosine was measured and percentage inhibition determined, as described in the text. Each data point represents an average of at least 2 experiments.

Little increases in inhibition were observed with increasing concentrations of inosine in the presence of adenine (Figure 6.8a). Inhibition progressed slowly and saturation was reached at 1 mM inosine, similar to the observations in the WT. Duplicate experiments were, however, not carried out on the WTAX parasites. EC_{50} value was 0.22 μM.

The effect of varying concentrations of Berenil® on uptake of 1 μM adenosine was also measured in the *T. evansi* isolate (WT). Results of inhibition are shown in Figure 6.9. In the absence of either inosine or adenine little or no inhibition was observed with Berenil® up to 100 μM concentration. Increased inhibition occurred at 500 μM. In the presence of 1 mM adenine there was no increase in inhibition with the addition of increasing concentrations up to 100 μM of Berenil®. However in the presence of 1 mM inosine a dose-dependent increase in inhibition was observed with Berenil®. Rapid increase in inhibition was observed above 10 μM, and highest inhibition at 100 μM was 73.5±22.6%). There was no saturation at this concentration of Berenil®. The EC_{50} value for this inhibition was 13.9 μM. Higher concentrations of Berenil® could not be used since Berenil®, is known to interfere with cell membranes of trypanosomes at concentrations above 100 μM (Barrett *et al.* 1995).

Similar observations on inhibition by Berenil® in the presence of inosine were made in the culture-adapted drug sensitive (WTAX) parasites.

6.3.3.2. *Drug resistant trypanosomes*

Results of adenosine uptake inhibition with increasing concentrations of inosine and adenine in the *in vivo*-derived resistant line (CR3) are shown in Figure 6.10a. Inhibition by increasing concentrations of adenine (in the presence of inosine) was rather slow, however, a dose-dependent effect was apparent. Little increase in inhibition occurred at 10 μM adenine and saturation was achieved at 50 μM adenine (97.4±0.2%). The EC_{50} value for this process was 4.0 ηM (inhibition by inosine alone in these parasites was over 75%). A more rapid dose-dependent increase in inhibition was observed with concentrations of inosine (in the presence of adenine). At 0.01-1 μM concentration of inosine, inhibition was quite slow (35.8-47.2%).

(This agreed with the low percentage inhibition by adenine in the drug resistant line (38.1%, Figure 6.2). However inhibition increased from 10 μM concentration (61.2±5.9%) and maximum inhibition at 100 μM was 92.1±0.4%. Complete saturation was not attained at 100 μM inosine. The EC_{50} value for this inhibition was 3.1 μM inosine.

The results of the interaction of Berenil® with the adenine/adenosine transporter in the CR3 is shown in Figure 6.10b. Relatively high percentage inhibition (up to 90%) was caused by 1 mM inosine in these parasites. A very slow increase in inhibition was observed with increasing concentrations of Berenil® in the presence of inosine starting from 89.9±0.9% at 0.01 μM to 94.6±0.4% at 100 μM Berenil®. This increase was much slower compared to the WT parasites. In the CR3 percentage inhibition remained unchanged at concentrations of Berenil® between 0.01-10 μM, in the presence of inosine. The EC_{50} value for this inhibition was 3.5 ηM.
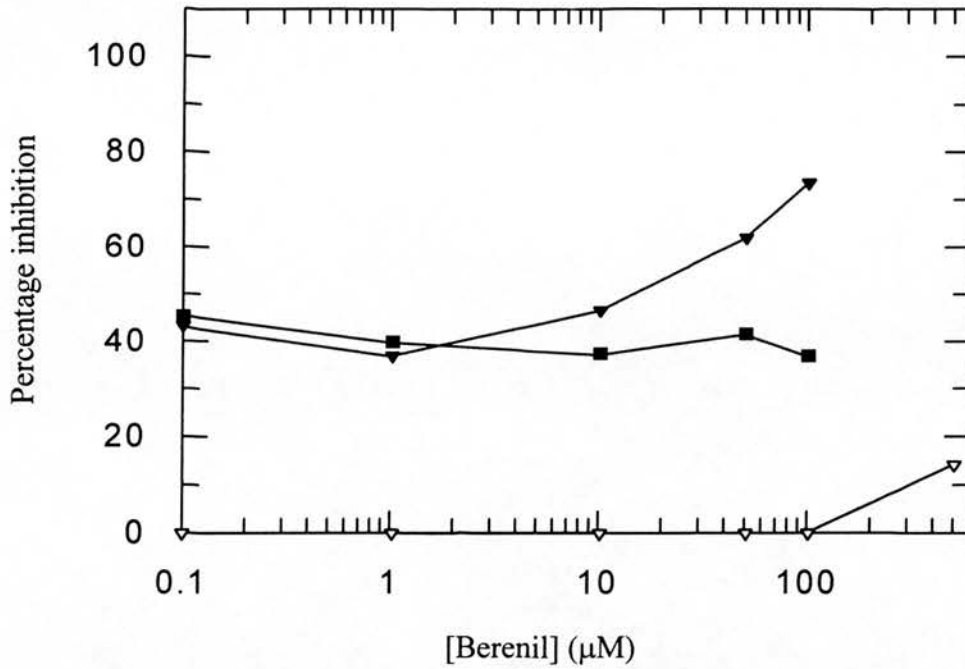
Figure 6.9: Dose-dependent effect of Berenil® on uptake of 1 µM adenosine in the *in vivo*-maintained drug sensitive *Trypanosoma evansi* (WT). Inhibition by Berenil® alone (open inverted triangles), Berenil® in the presence of 1 mM inosine (filled inverted triangles) or Berenil® in the presence of 1 mM adenine (filled squares). Adenosine uptake assays were carried out and percentage inhibition of uptake calculated as described in the text. Each data point represents average of at least 3 experiments.
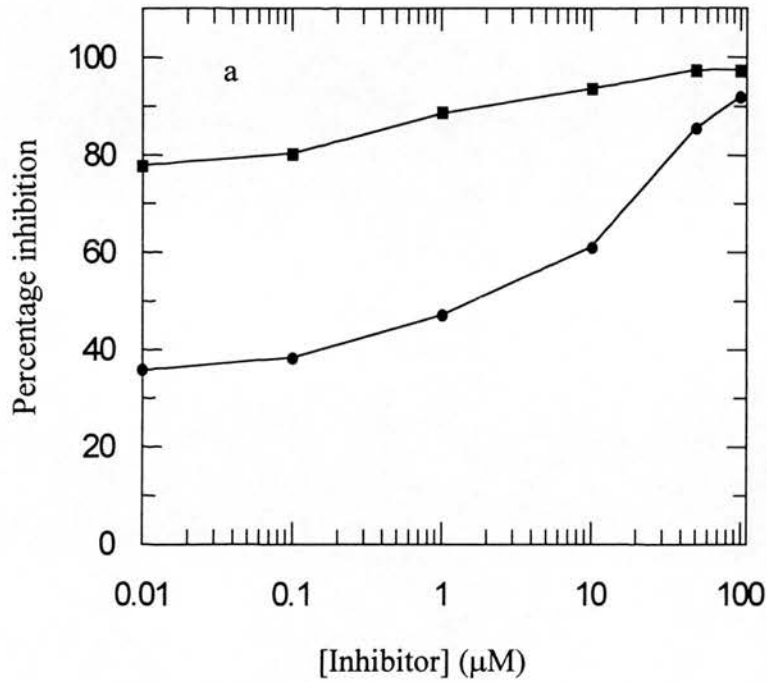
Figure 6.10: Inhibition of uptake of 1 µM adenosine in the *in vivo*-derived drug resistant *Trypanosoma evansi* (CR3). a, inhibition by inosine in the presence of 1 mM adenine (filled circles), and adenine in the presence of 1 mM inosine (filled squares); b: inhibition by Berenil® in the presence of 1 mM inosine. Each data point represents average of at least 2 experiments.
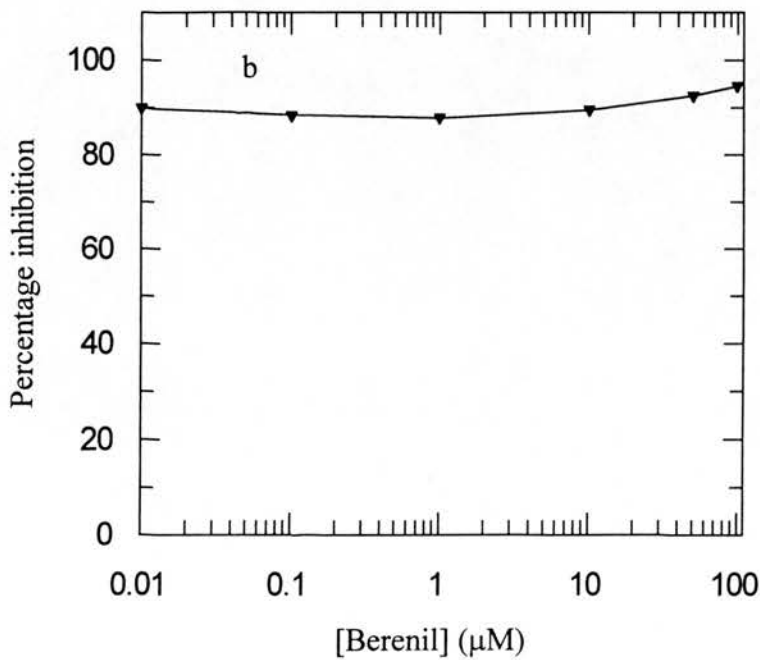
6.4. DISCUSSION

From this study, uptake of 1 μM adenosine was completely inhibited in the presence of saturating concentrations of both adenine and inosine. This suggests that *T. evansi* TREU 1840 possesses two adenosine transport systems, one inhibitable by adenine and the other by inosine. This observation is in agreement with the observations of Carter and Fairlamb (1993) who showed that *T. brucei* possessed two adenosine transporters, also inhibited by adenine and inosine respectively. Carter and Fairlamb (1993) named these transporters P1 (inhibitable by inosine) and P2 (inhibitable by adenine). Thus *T. evansi* also possess the P1/P2 bipartite adenosine transport system. Also similar to the observations of Carter and Fairlamb, this study showed that in *T. evansi* the P1 transporter can be saturated by increasing concentrations of inosine up to 1 mM and the P2 similarly saturated by increasing concentration of adenine up to 100 μM. Observations of P1/P2 bipartite adenosine transporters have also been made on *T. equiperdum* (Barrett *et al.*, 1995). Scott *et al.* (1997), however, found that adenosine transport in bloodstream forms of a strain of *T. brucei* (247) could only be partially inhibited by both adenine and inosine. He suggested at least four adenosine transport systems for the *T. brucei* strain: one transporting adenosine and inosine, the second adenosine and adenine (including mel Cy and mel Ox), the third adenosine, adenine and inosine and the fourth adenosine alone.

The existence of two purine nucleoside transporters with non-overlapping substrate specificities was reported in *Leishmania donovani* promastigotes (Aronow, *et al.*, 1987), the first transported inosine, guanosine and their analogues while the second transported adenosine, analogues of adenosine, and the pyrimidine nucleosides, uridine, cythidine and thymidine. In the case of *T. evansi,* as shown in the present study, the P1 and P2 adenosine transporters have overlapping substrate specificities in that both the P1 and the P2 transport adenosine, in addition to either inosine or adenine. The results on the WT parasites indicate that there may also be some level of overlap in the substrate specificity for inosine and adenine on either the P1 and/or P2 transporters. This can be appreciated from the observation that both the percentage inhibitions by inosine (23.2%) and adenine (53.6%) taken individually

only added up to 76.8%. However, uptake in the presence of inosine (76.8%) and adenine (46.4%) added was in excess (123.2%) showing that inhibition was not complete. It is possible that one of the transporters mediates for uptake of both inosine and adenine. In that case it is not likely that this would be the P2 because inosine did not inhibit mel Cy lysis, mel Cy being a substrate for the P2. Thus the P1 probably transports adenine to a limited extent.

The existence of two distinct adenosine transporters have been reported in other members of Trypanosomatidae (James and Born, 1980; Baer *et al.*, 1992).

The present study showed that the P1 was a smaller adenosine transport system in *T. evansi* compared to the P2. Carter and Fairlamb (1993), found the opposite to be true in *T. brucei*: Inosine inhibited 60-70% while uptake via the P2 accounted for only 30-40% of total adenosine uptake, indicating that the P1 was the more elaborate process. Scott *et al.* (1997) observed maximum inhibitions of 45% and 50%, respectively, with saturating concentrations of inosine and adenine, suggesting that the capacities of the two transporters were fairly similar in that strain of *T. brucei*. These differences in activity by the P1 and P2 transporters in different species and strains of trypanosomes further amplify the variations that exists in purine transport functions within the Trypanosomatidae.

That adenine was a more potent inhibitor of adenosine transport in drug sensitive *T. evansi* was also evident from the effects of increasing concentrations of these inhibitors on uptake. Possible explanations for the differences in the apparent potency of inhibition between inosine and adenine are two-fold. Either the P1 transporter has lower affinity for inosine and the P2 has a higher affinity for adenine, or there may be more binding sites or transporter molecules for adenine than there are for inosine. This remains to be verified.

The lower EC_{50} value for inosine in the presence of adenine (0.22 μM) compared to that of adenine in the presence of inosine (0.83 μM) in the WTAX parasites may be due to higher inhibitions caused by adenine in these parasites and not singularly the effect of inhibition by inosine alone. Low concentrations giving 50% inhibition of

transport (IC_{50}) at the P2 by adenine (0.16 µM) have also been reported in *T. equiperdum* (Barrett, *et al.*, 1995).

The maximum inhibition of adenosine uptake caused by deoxyadenosine confirm that the structure recognised by these adenosine transporters is not the carbohydrate but the purine part of the molecule. It has been suggested that the melaminyl moiety targets the melaminophenyl arsenicals into drug sensitive trypanosomes via the P2 transporter (Fairlamb *et al.*, 1992b; Carter and Fairlamb, 1993). This means the melamine ring is recognised similar to the purine ring.

From the present study Berenil® caused inhibition of adenosine uptake in a dose-dependent manner similar to those caused by inosine and adenine. However high concentrations of Berenil® were required to produce a dose-dependent inhibition in the absence of inosine or adenine. Increased inhibition by Berenil® in the presence of inosine in the WT and WTAX parasites confirm that Berenil® interacted with the P2. Thus Berenil® and possibly other diamidine drugs are taken up into drug sensitive *T. evansi* by the P2 adenosine transporter. This was also evident from the lysis data in which Berenil® blocked lysis of trypanosomes by mel Cy similar to adenine and adenosine , while inosine did not block lysis.

Similar observations on interaction of diamidines with adenosine transporters have been reported in *T. brucei* (Scott *et al.*, 1997; Berger *et al.*, 1995; Carter *et al.*, 1996), *T. evansi* (Ross and Barns, 1996) and *T. equiperdum* (Barrett *et al.*, 1995. The observation of a common mechanism of transport between Berenil® and mel Cy in this study confirms the basis of cross resistance between the diamidines and some melaminophenyl arsenicals, but not mel B. An alternative mechanism of uptake not involving adenosine transport may be responsible for uptake of mel B.

This study has demonstrated that the P2 transporter is conserved in arsenical resistant *T. evansi.*

The two transport systems were maintained in the drug resistant *T. evansi* although with modifications in the activity. Up to 10-fold decrease in P2 and 3-fold increase in the P1 were observed, with up to 45% reduction in overall uptake. Alterations in

P2 transport leading to reduced accumulation of pentamidine has also been reported by Carter *et al.* (1996). The events that led to differential decreases in P2 activity in the different resistant trypanosome lines observed in this study are not well understood. The reductions in uptake by the P2 are thought to be responsible for the decrease in overall uptake of adenosine by these parasites. There appears to be some compensatory increase in percentage uptake at the P1 transporter in all the drug resistant lines. This however, was not enough to bring the total adenosine uptake to similar levels as the drug sensitive trypanosomes.

The reduction in uptake at the P2 could be a result of mutations leading to conformational changes in the binding sites on the transporter molecules leading to reduced affinity for substrate. Alternatively a reduction in the number of transport or carrier molecules produced in the resistant trypanosomes may be responsible for the observed resistant phenotype. For this to be established detailed kinetic study of the P2 transporter is required.

Observations on the conservation of P2 adenosine transporter in Berenil-resistant *T. equiperdum* was reported by Barrett *et al.* (1995). These authors proposed that loss of affinity for substrate was responsible for the reduced uptake by the P2 transporter in *T. equiperdum*. However, the authors were not able to determine the K_m and V_{max} values for the P2 transporter of the Berenil®-resistant *T. equiperdum*. Their conclusions were based only on reduced uptake of adenosine from inhibition studies, as well as the expression of limited resistance to drugs using drug assays. If the P2 transport is still present in drug resistant trypanosomes then determination of kinetic properties of this transporter is required in order to identify the type of mutation responsible for the reduced capacity for transport.

The finding that the P2 transporter is still present in the drug resistant trypanosomes could explain our observations on lysis of drug resistant trypanosomes at relatively low concentrations of mel Cy. (see chapter 5). This confirms that mel Cy resistant trypanosomes retain some ability to transport melaminophenyl arsenicals. This may account for their sensitivity to moderately increased concentrations of drug.

It is not completely clear whether the retention of variable levels of the P2 transporter is responsible for the differences in stability of resistance following prolonged withdrawal of drug. (see chapter 4). The instability of resistance in the *in vivo*-derived CR3.1 which was found to possess more P2 activity compared to the *in vitro*-derived MCR1.1 with less P2 activity suggests this.

There was evidence that Berenil$^{®}$ still interacted with the P2 transporter in the *in vivo*-derived resistant lines, but not in the two *in vitro*-derived resistant lines. Since the *in vitro*-selected resistant trypanosomes had least of the P2 activity it suggests that lack of further inhibition by Berenil$^{®}$ may be a reflection of the extent of reduction in P2 in these parasites.

Contrary to the observations on the drug sensitive trypanosomes, combination of Berenil$^{®}$ and adenine in the resistant trypanosomes resulted to increased inhibition. Since Berenil$^{®}$ showed no interaction with the P1 in the drug sensitive parasites it is reasonable to expect that the observed increase in inhibition in the presence of adenine was not due to an effect on the P1. It is possible that the P2 transporter has been drastically modified and now interacts with Berenil$^{®}$ in a different way. Further work needs to be done to elucidate the specific changes occurring at the P2 and any other transporters that give rise to arsenical resistance in *T. evansi*.

This study has compared the use of *in vitro*-derived drug resistant trypanosomes with the *in vivo*-derived resistant lines in the mechanisms of drug resistance. It was observed that the *in vitro*-derived drug resistant lines produced very similar results to their corresponding *in vivo*-derived resistant lines. This substantiates the fact that *in vitro* systems can to a great extent be successfully used to replace the use of animal models in the study of drug resistance.

CHAPTER SEVEN

KINETICS OF ADENOSINE TRANSPORT AND INHIBITION IN DRUG SENSITIVE AND DRUG RESISTANT *TRYPANOSOMA EVANSI*

7.1. INTRODUCTION

Passage of nucleosides across plasma membranes may occur by simple diffusion or may be carrier-mediated. Simple diffusion has been found to account for a variable proportion of nucleoside movement across cell membranes. It is recognisable experimentally as its rate is directly proportional to the nucleoside concentration gradient and is unaltered by the presence of other substrates or nucleoside transport inhibitors (Clanachan *et al.*, 1987). Carrier-mediated transport occurs either by facilitated diffusion or by active transport, each involves specific nucleoside transport processes (Cass, 1995).

Nucleoside transport processes have been divided into two distinct classes, the equilibrative and the concentrative transport processes. The equilibrative nucleoside transport process exhibits the typical features associated with facilitated diffusion. It is driven by the concentration gradient of the nucleoside being transported. They function in both uptake and release of nucleosides from cells. The concentrative nucleoside transport processes are secondary active systems that are driven by transmembrane Na^+ gradients and, in some cases are inwardly directed Na^+-nucleoside cotransporters. Equilibrative nucleoside transport processes are ubiquitous among mammalian cells and tissues. The concentrative nucleoside transport processes are limited to specialised cell types such as intestine, kidney, spleen, lymphocytes, macrophages and choroid (Cass, 1995).

Mammalian cells as well as parasites require the presence of nucleoside transport proteins in their plasma membranes for the uptake (or release) of physiological nucleosides. The process by which these purine nucleosides permeate the plasma membranes independent of intracellular fate such as binding or metabolism is

described as nucleoside transport (Aronow *et al.*, 1987; Ogbunude and Baer, 1993). The term uptake, on the other hand, is used to represent both transport across cell membrane and the accumulation of the substrate and its metabolic products within the cell (Kidder *et al.*, 1978; Ogbunude and Baer, 1993).

In protozoans uptake of purine nucleosides is generally assumed to occur via facilitated diffusion (Aronow *et al.*, 1987). Although recently, it was shown (Ogbunude and Dzimiri, 1993) that in *L. donovani* promastigotes adenosine is not only transported by facilitated diffusion but also by a channel-like pathway.

Studies on purine transport in trypanosomes so far have provided evidence for the presence of two adenosine transporters in drug sensitive trypanosomes. These were identified as P1 (which also transports inosine) and P2 (which also transports adenine) (Carter and Fairlamb, 1993). Subsequent reports, however, suggest that there may be more than two adenosine transporters in trypanosomes (Ross and Barns, 1996; Scott *et al.*, 1997).

The P2 transporter has been associated with transport of melaminophenyl arsenicals into drug sensitive trypanosomes. Loss of this transporter in an arsenical resistant *T. brucei* was proposed as a mechanism of resistance to these drugs (Carter and Fairlamb, 1993).

The present study however, has demonstrated that the P2 transporter is retained in arsenical resistant *T. evansi*, although its capacity for uptake of substrate was greatly decreased.

It is not understood whether the reduced uptake observed at the P2 transporter of the arsenical resistant *T. evansi* was due to decreased affinity for substrate or a reduction in numbers of the transporter molecules. To address this question this study was undertaken with the following specific objectives.

1. To determine the kinetic properties of both the P1 and the P2 adenosine transporters in drug sensitive *T. evansi*, as this has not been done before.

2. To determine how the properties of the P1 and P2 adenosine transporters are altered in drug resistant trypanosomes, selected using a melaminophenyl arsenical drug, mel Cy.

3. To determine how the changes in kinetic properties of the P2 transporter affect alternative substrates taken up by this transporter.

An investigation of possible accompanying changes at the P1 transporter may further elucidate any adaptive mechanisms which enable drug resistant trypanosomes evade the toxic effects of drugs while at the same time maintaining their normal physiological functions. These could be exploited for therapeutic purposes.

7.2. MATERIALS AND METHODS

7.2.1. Trypanosomes

The trypanosomes used in this experiments consisted of the mouse-maintained drug sensitive(WT) parasites, and its *in vivo*-derived drug resistant clone, CR3.1.

7.2.2. Kinetic studies of adenosine transport I : Time course

7.2.2.1. Adenosine transport assays

Transport of 5 μM adenosine was measured at different time points between 10 and 120 seconds, in drug sensitive and drug resistant trypanosomes. Transport activity on either the P1 or P2 transporter was determined by inhibition of one transport process with a saturating concentration of either adenine or inosine, respectively. Transport was also measured in the absence of inhibitor.

Trypanosomes separated from mouse blood were washed twice in PSG and resuspended in transport buffer (CBSS, consisting of 25 mM Hepes/ 120 mM NaCl/ 5.4 mM KCl/ 0.55 mM $CaCl_2$/ 0.4 mM $MgSO_4$/ 5.6 mM Na_2HPO_4/ 11.1 mM glucose, pH 7.4; Fairlamb *et al.*, 1992a), at a density of 2.5×10^8 trypanosomes/ml, and kept on ice. Trypanosome suspensions were prewarmed to 37°C for 5 minutes.

Aliquots of 80 µl (containing 2×10^7 trypanosomes) were added to each of a series of reaction tubes held at 37°C on a water bath. Inhibitors at 50x final concentration in assay were added in 2 µl volumes to appropriate tubes. Tritiated adenosine was prepared at 5 times its final concentration of 5 µM in assay. At zero time 20 µl of [2-^{3}H]-adenosine (specific activity 185 Bq/mmol or 12.5 mCi/mmol) was added to the reaction medium using a multiple drop Finn pipette. Experiments were timed carefully. At the end of incubation 100 µl of Stop solution, consisting of ice-cold 5 mM non-radiolabelled adenosine in PBS, was added to the reaction medium. The mixture was quickly transferred to an Eppendorf filter unit (SPIN-X, COSTAR) containing 0.45 µm acetate filter membrane. The filter units were centrifuged at maximum speed (100x g) for 40 seconds in a biofuge. The filters were washed with 0.5 mls of Stop solution and centrifuged at 100x g for 1 minute. The top of the filter unit was snipped off using a pair of scissors, the filter holder was removed and the bottom part containing the filter membrane was cut off using a "micro cutter". The filter membranes were placed in scintillation vials and dried over night at 50°C (or for 1.5 hours at 100°C). One ml of scintillation fluid (PACKARD, U.K) was added to each scintillation vial and incorporated radioactivity counted in a scintillation counter.

Control incubations (blanks) were performed in the absence of trypanosomes to correct for non-specific binding of radioactivity. The data was corrected for this value in each case. Triplicate tubes were used for each assay and each experiment was repeated 3-4 separate times.

Uptake of adenosine was calculated from counts (counts per minute, CPM) of incorporated radioactivity, and expressed as ρmoles/10^8 trypanosomes.

Uptake data was analysed using non-linear regression analysis programme, using MICROCAL ORIGIN computer software. The following exponential association equation was used to estimate the various uptake parameters as illustrated in Figure 7.1.
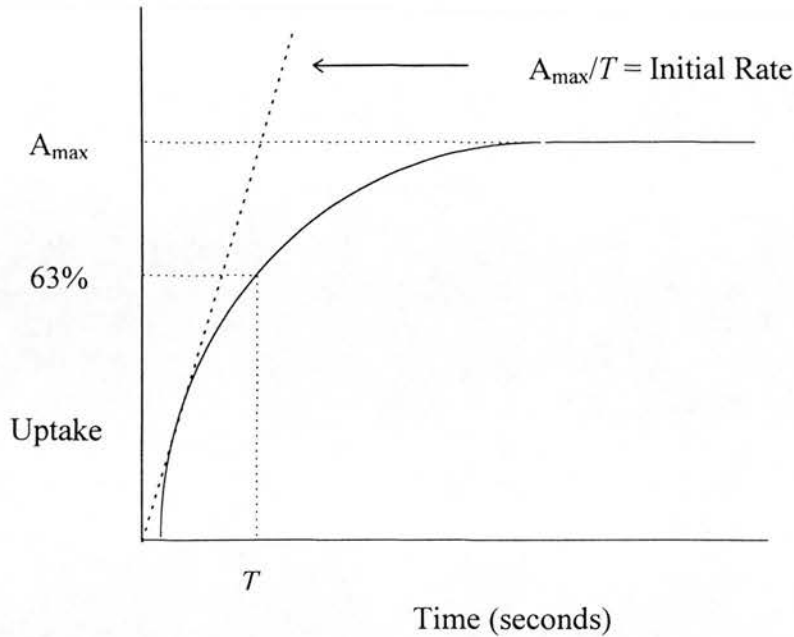
Figure 7.1: Illustration of the method of estimation of adenosine uptake rate parameters in *T. evansi* by the MICROCAL ORIGIN software.

$y = y^0 + A_{max1} (1-e^{-x/T1}) + A_{max2} (1-e^{-x/T2})$

Where

y = uptake (ρmoles/10^8 trypanosomes), one uptake process was assumed to be zero

x = time (seconds)

T = rate constant = the time (seconds) when 63% of the maximum concentration (A_{max}) is attained

A_{max} = maximum concentration at time infinity (ρmoles/10^8 trypanosomes)

y^0 = correction if t_0 is not zero

From these plots initial transport rates were calculated from the equation

$C_t = cP_{max}. (1-e^{-x/T}) = y = A_{max} (1- e^{-x/T})$

Gradient = cP_{max}/T or $A_{max}/Te^{-x/T}$

At time zero gradient = cP_{max}/T or A_{max}/T

Therefore,

A_{max}/T = Rate of change in concentration of adenosine, hence measures initial uptake rate.

Attempts were made to separate the individual transport processes (P1 and P2) using this programme from data on the experiments performed in the absence of inhibitor.

Transport data was analysed using two-sample T-test.

7.2.3. Kinetic studies of adenosine transport II: Effects of changes in substrate concentration on velocity of uptake

7.2.3.1. Uptake assays

Trypanosomes separated from mice blood were washed by centrifugation at 100x g for 6 minutes in a biofuge. The trypanosome pellet was resuspended in transport buffer at a density of 1.25×10^8 trypanosomes/ml. Trypanosome suspensions were prewarmed to 37°C for 5 minutes. Aliquots of 80 µl (containing 1×10^7 trypanosomes) were added to each of a series of reaction tubes on a water bath at 37°C. Inhibitors, consisting of 1 mM adenine (to block the P2 and measure the uptake at P1) or 1 mM inosine (to block the P1 and measure the uptake at P2) were added at 50 times the final concentration in assay, in volumes of 2 µl. Tritiated adenosine was included in the reaction mixture at the following concentrations 0.1, 0.25, 0.5, 0.75, 1.0, 2.5, 5.0, or 10.0 µM (each at 5 times final concentration). Experiment was started by the addition of 20 µl of the appropriate concentration of $[2-^3H]$ adenosine. Assay tubes were incubated for 5 minutes at 37°C. The reaction was stopped by addition of 100 µl of 1 mM unlabelled adenosine in PBS (Stop solution). The reaction mixture was immediately transferred to an Eppendorf filtration unit containing 0.45 µm acetate filter (SPIN-X, COSTAR) and centrifuged at 100x g for 40 seconds in a biofuge. The filters were washed once with 0.5 ml of Stop solution and again centrifuged at 100x g for 1 minute. The top part of the filter holder was cut off using a micro cutter and the filters were placed in scintillation vials and dried overnight at 50°C. One ml of scintillation fluid (PACKARD, UK)

was added to each scintillation vial and incorporated radioactivity counted in a scintillation counter. Uptake of adenosine was calculated from counts of decompositions of incorporated radioactivity, and expressed as pmoles/10^8 trypanosomes.

Control experiments were performed in absence of trypanosomes (blanks) to correct for any non-specific binding of radioactivity. The data was corrected for this value in each case. Duplicate tubes were used for each assay. Each experiment was repeated 3-4 separate times.

The uptake data obtained from these experiments was fitted to rectangular hyperbolic plots and analysed using non-linear regression analysis programme. Kinetic parameters were determined using Michaelis-Menten equation from MICROCAL ORIGIN computer software, as shown in Figure 7.2.
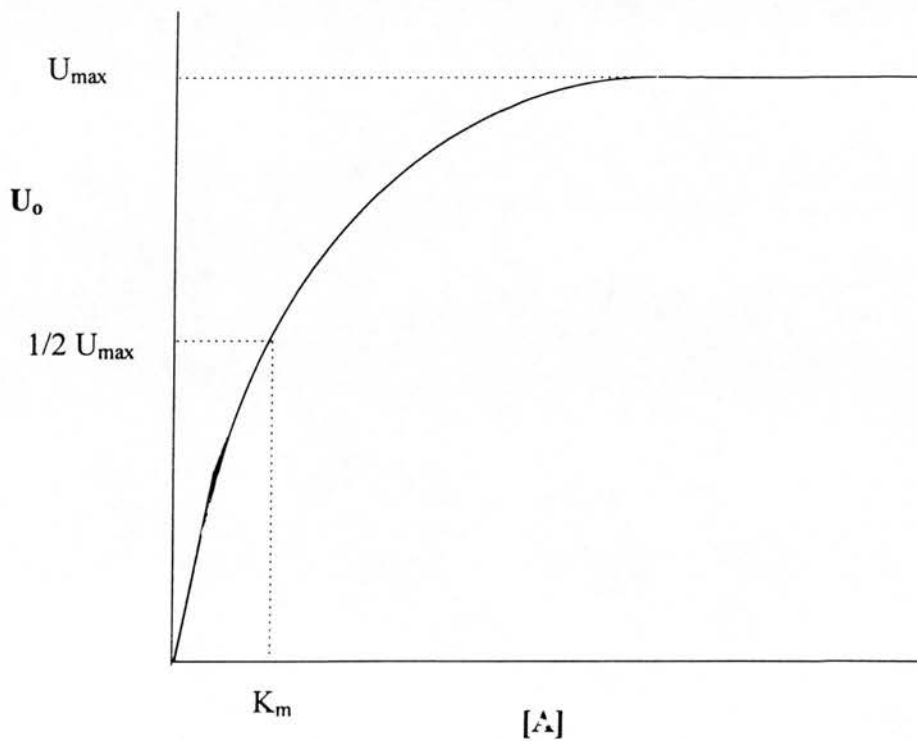
Figure 7.2: Illustration of the method of estimation of Michaelis-Menten kinetic parameters of adenosine uptake in *T. evansi* by the MICROCAL ORIGIN computer software.

$$U_o = U_{max} \cdot [A] / ([A] + K_m)$$

where

U_o = initial velocity of uptake (ρmoles/10^8 trypanosomes/5minutes)

U_{max} = maximum velocity of uptake = value of U_o when $[A]$ = ∞ (ρmoles/10^8 trypanosomes/5minutes). U_{max} represents the maximal rate at which a transporter functions. It is attained when the transporter is saturated with substrate.

$[A]$ = substrate (adenosine) concentration (μM).

K_m = dissociation constant (μM) = value of $[A]$ when U_o = ½ U_{max}. The K_m value of a transporter indicates the concentration of substrate at which half the active sites are filled. It represents the strength with which a transporter binds to its substrate (Cohn and Gottlieb, 1997).

In this presentation the term U_{max} has been used instead of the conventional V_{max} in Michaelis-Menten kinetics, since enzymes were not being used.

Plots of the data collected revealed the presence of a non-carrier mediated (passive) diffusion process (constituting a linear component in the uptake curves). This became prominent at concentrations above 5 µm adenosine. A modified Michaelis-Menten equation employing a hyperbolic and a linear component was then adopted which effectively separated the passive diffusion component from the carrier-mediated uptake by the P1 and P2 transporters, as illustrated in Figure 7.3.
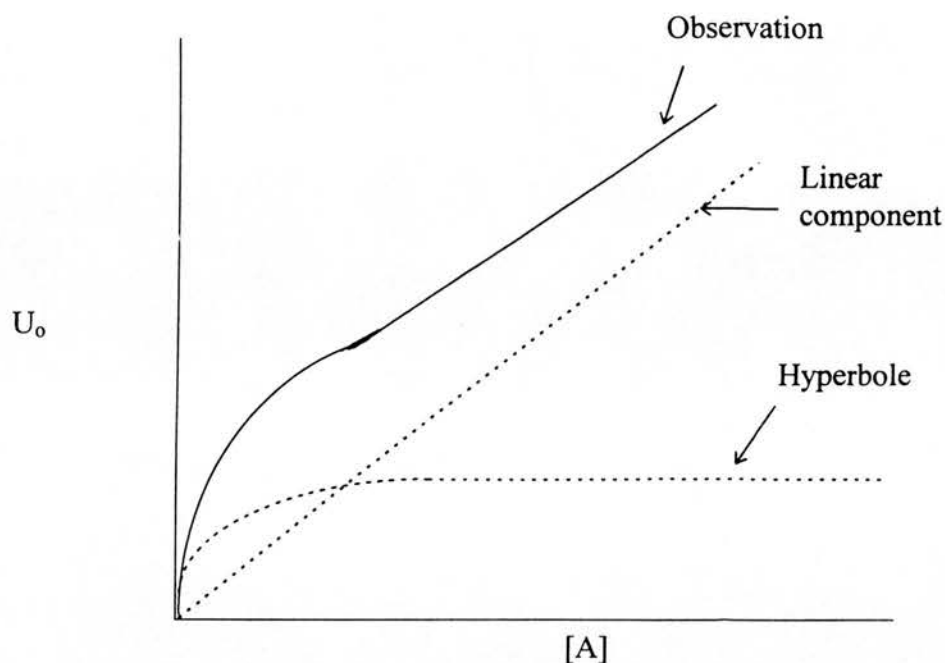
Figure 7.3: Illustration of the method of correction for adenosine uptake due to passsive diffusion (linear component) by the MICROCAL ORIGIN software.

$$U_o = (U_{max} \cdot [A] / ([A] + K_m)) + K_L \cdot [A]$$

Where,

K_L = rate constant of the linear uptake (passive diffusion)

Data obtained from each experiment was corrected for the passive diffusion component using the modified Michaelis-Menten equation. The programme estimated the gradient for the linear component and subtracted it from the rest of the uptake as shown above.

7.2.4 Kinetics of inhibition of adenosine uptake in *Trypanosoma evansi*

Kinetics of inhibition of adenosine uptake by inosine, adenine and Berenil® were also studied in both drug sensitive (WT) and drug resistant (CR3.1) trypanosomes.

Effect of increasing concentration of either inosine, adenine or Berenil® on inhibition of adenosine uptake was determined by measuring the uptake of 1 µM tritiated

adenosine by 10^7 trypanosomes, at 37°C, for 5 minutes. Uptake was measured in the presence of increasing concentrations of each inhibitor. The effect of inhibition at the P1 or P2 transporters was determined by saturating one transporter with either 1 mM inosine or 1 mM adenine.

Adenosine uptake assays were carried out as described above (section 7.2.4.1.) The concentrations used for inosine, adenine and Berenil® were 0.01, 0.1, 1, 10, 50, 100 & 1000 μM; 0.01, 0.1, 1, 10, 50 & 100 μM; and, 0.01, 0.1, 1, 10, 50, 100 & 500 μM; respectively.

The uptake data obtained was plotted and analysed using non linear regression analysis programme. Inhibition Constants were estimated using the equation below.

$U_o = U_{max} \cdot [S] / ([S] + K_m (1 + I/K_i))$

$= U_{max} \cdot 1 / (1 + K_m (1 + I/K_i))$

$= U_{max} / (1 + K_m (1 + I/K_i))$

Where,

U_o = initial velocity of uptake of adenosine (ρmoles/10^8cells/5minutes)

U_{max} = maximum initial velocity of uptake of adenosine (ρmoles/10^8cells/5 minutes)

$[S]$ = concentration of substrate (adenosine, μM)

K_m = dissociation constant for adenosine on the P1 or P2 (μM) (= fixed)

I = concentration of inhibitor (μM) (varies from 0.01 - 1000 μM)

K_i = dissociation constant for inhibitor (μM)

By substitution,

$y = a + b/((1 + x/c) \cdot d + 1))$

Where

$a = 0$ (fixed)

$b = U_{max}$

$c = K_i$

x = I

d = K_m (fixed as 0.07, 0.74 µM for P1 and P2, respectively in drug sensitive trypanosomes; and 0.06, 0.1 µM for P1 and P2 in drug resistant trypanosomes.

Data was analysed using two-sample T-test.

7.3. RESULTS

7.3.1. Kinetic studies of adenosine transport I: Uptake rates

Transport of 5 µM adenosine via the P1 and P2 transporters was measured over a 120 second duration in the presence of saturating concentrations of either adenine or inosine. The total (overall) uptake represented both processes and was measured in the absence of inhibitor. Adenosine transport curves for drug-sensitive trypanosomes are shown in Figures 7.4A-D. Transport in drug resistant parasites is shown in Figures 7.5A-D.

Similar to results from adenosine uptake inhibition studies there was no transport in the presence of saturating concentrations of both adenine and inosine in either the drug sensitive or drug resistant trypanosomes (Figures 7.4D and 7.5D). This confirms that *T. evansi* TREU 1840 possessed two adenosine transport systems.

Kinetic parameters, including maximum concentration at time infinity (A_{max}), the rate constant (T) and the initial rates of transport, estimated from exponential plots are shown in Table 7.1.

The A_{max} for the total uptake of adenosine (in the absence of inhibitor) in the drug sensitive (WT) trypanosomes was 140.6±24.0 pmoles/10^8trypanosomes. This represents the total capacity of uptake by the P1 and P2 transport processes. The
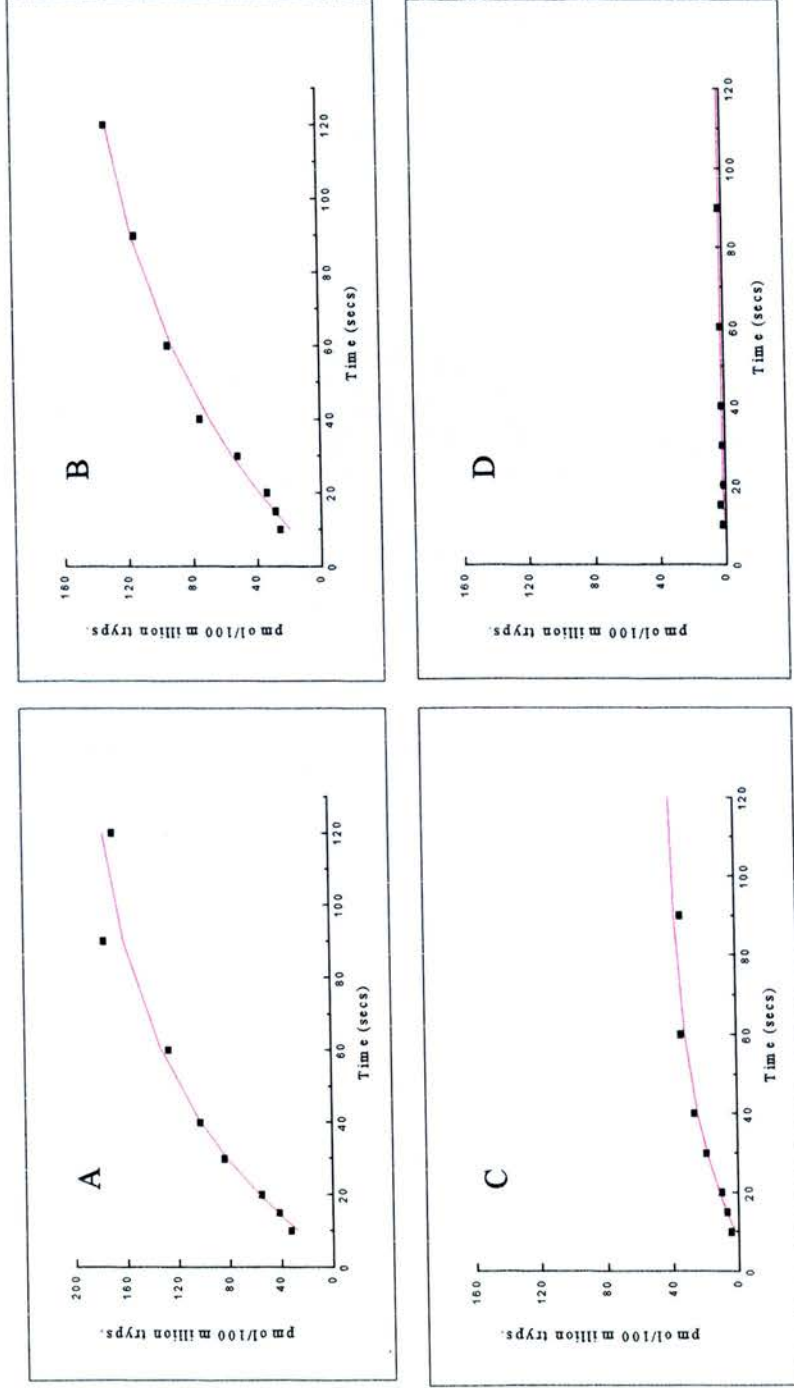
Figure 7.4: Adenosine transport in drug sensitive *Trypanosoma evansi* (WT). I : Time course. 2x 10^{7} trypanosomes were incubated with 5 μM tritiated adenosine either in the absence of inhibitor (A); or presence of 1 mM inosine (B), 1 mM adenine (C), or 1 mM of both adenine and inosine (D); at 37°C. Incorporated radioactivity was measured at various time points between 10 and 120 seconds. Trypanosomes were separated from transport medium by filtration and centrifugation through 0.45 μm cellulose filter (COSTAR). Uptake was calculated from counts of decompositions of radioactivity recorded from a scintillation counter; and expressed as pmoles/10[8] trypanosomes. Data was fitted to exponential plots using non-linear regression analysis programme. From these fittings transport rates were estimated as described in the text. Each experiment was repeated 3 or 4 times and similar results were obtained.
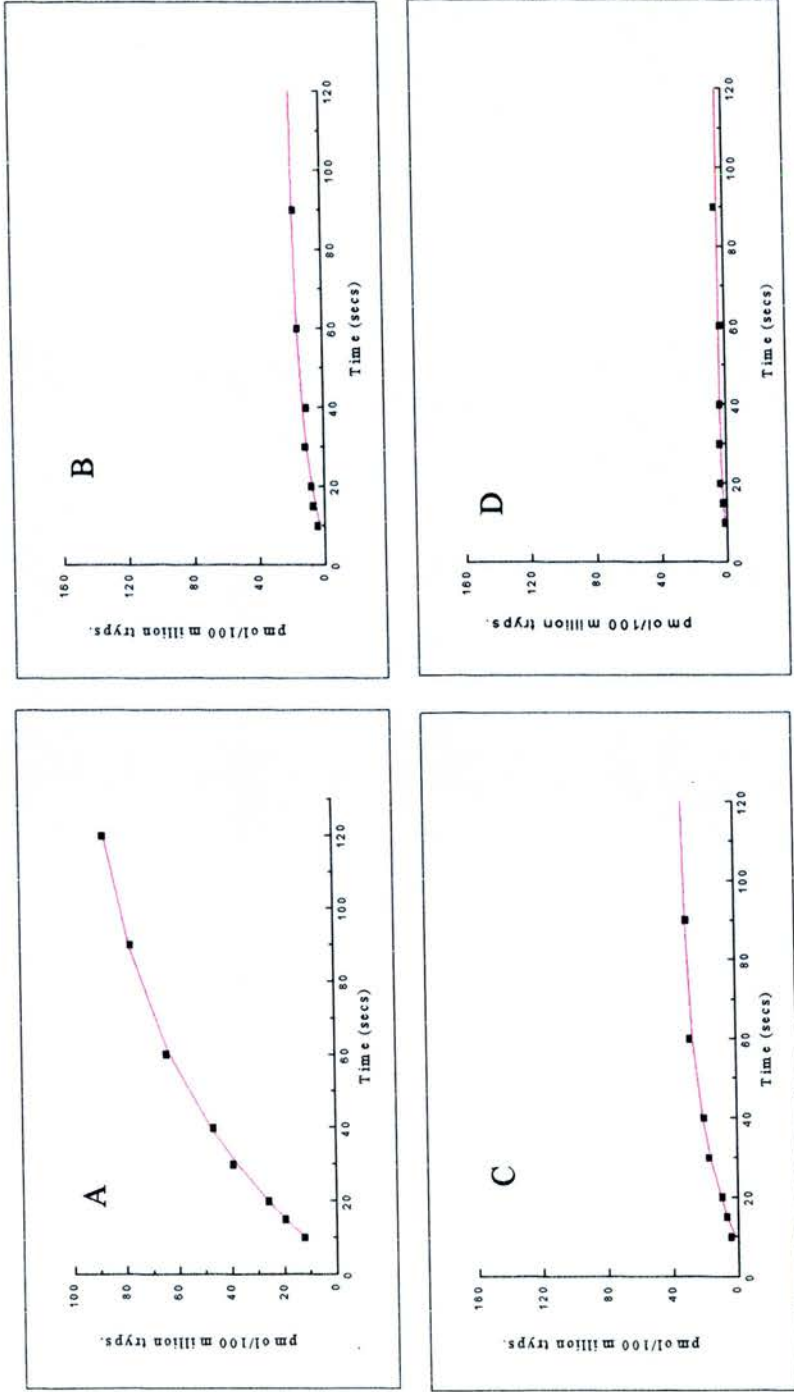
Figure 7.5: Adenosine transport in drug resistant *Trypanosoma evansi* (CR3.1). I: Time course. 2×10^7 trypanosomes were incubated with 5 µM tritiated adenosine either in the absence of inhibitor (A); or presence of 1 mM inosine (B), 1 mM adenine (C), or 1 mM of both adenine and inosine (D); at 37°C. Incorporated radioactivity was measured at various time points between 10 and 120 seconds. Trypanosomes were separated from transport medium by filtration and centrifugation through 0.45 µm cellulose acetate filter (COSTAR). Uptake was calculated from counts of decompositions of radioactivity recorded from a scintillation counter; and expressed as pmoles/10^8 trypanosomes. Data was fitted to exponential plots using non-linear regression analysis programme. From these fittings transport rates were estimated as described in the text. Each experiment was repeated 3 or 4 times and similar results were obtained.

rate constant for this uptake was 57.7 ± 10.3 seconds. The initial rate was 2.67 ± 0.53 pmoles/second/10^8trypanosomes.

In the drug resistant clone there was a slight decrease (not statistically significant: T = 0.66, P = 0.55, N = 4) in the overall uptake capacity. A_{max} for the overall uptake (in the absence of inhibitor) was 122.6 ± 13.0 pmoles/10^8 trypanosomes. The rate constant was slightly increased to 72.8 ± 9.2 seconds representing only a slight (not statistically significant: T= -1.09, P= 0.32, N= 4) reduction in the speed of the overall transport process. The initial rate of the overall transport in the drug resistant clone (1.78 ± 0.3 pmoles/10^8trypanosomes) was not significantly different (T= 1.49,P=0.21, N= 4) compared to the WT trypanosomes. It was not possible from this data to separate the individual (P1 and P2) transport processes using data on overall uptake in the absence of inhibitor.

In the wild type (WT) the P2 was found to be the larger component of the two transport processes. The A_{max} for the P2 transporter was 112.5 ± 17.8 pmoles/10^8 trypanosomes. This represent approximately 80% of the total uptake (with no inhibitor). The rate constant on the P2 transporter was 71.2 ± 9.7 seconds being higher than the rate constant for the overall process (57.7 seconds). The initial rate for this transporter was 1.63 ± 0.3 pmoles/sec/10^8 trypanosomes. The initial rate was lower than the initial rate (2.67 ± 0.5 pmoles/sec/10^8 trypanosomes) of the overall uptake in absence of inhibitor.

The A_{max} of the P2 transporter in the drug resistant trypanosomes was significantly reduced (T=4.01, P=0.016, N=4) with a mean of 33.6 ± 8.5 pmoles/10^8trypanosomes. This represents only 27.4% of the overall uptake capacity in these trypanosomes, in contrast to 80% contribution to the total uptake, in the drug-sensitive parasites. The Rate constant was also reduced in the drug-resistant trypanosomes with a value of 39 ± 9.0 seconds, although the difference was not statistically significant (T= 2.44, P=0.059, N= 4). This rate constant had also reduced compared to the rate constant of the overall uptake (72.8 ± 9.2 seconds) in the same drug-resistant trypanosomes. This means the speed of activity of the P2 transporters in the drug-resistant trypanosomes was increased but the uptake capacity was decreased. The initial rate of transport for

the P2 transporter in the drug resistant population was 0.81 ± 0.3 pmoles/sec/10^8 trypanosomes. Although this represents a 2-fold reduction compared to the sensitive population it was not found to be statistically significant (T=2.21, P=0.078, N=4).

Table 7.1: Kinetic parameters of adenosine transport in drug sensitive and drug resistant *Trypanosoma evansi*. I: Uptake rates.

Adenosine transport	Drug sensitive (WT)			Drug resistant (CR3.1)		
	A_{max} (pmol/10^8 cells)	Rate constant (T, sec)	Initial Rate (pmol/10^8 cells/sec)	A_{max} (pmol/10^8 cells)	Rate constant (T, sec)	Initial rate (pmol/10^8 cells/sec)
Total uptake	140.6 ± 24.0	57.7 ± 10.3	2.67 ± 0.5	122.6 ± 13.0	72.8 ± 9.2	1.78 ± 0.3
Adenine present (P1)	67.7 ± 9.7	60.4 ± 9.0	1.26 ± 0.2	43.9 ± 2.0	19.3 ± 5.8	2.68 ± 0.7
Inosine present (P2)	112.5 ± 17.8	71.2 ± 9.7	1.63 ± 0.3	33.6 ± 8.5	39.0 ± 9.0	0.81 ± 0.3

Legend: A_{max} = maximum concentration at time infinity
Rate constant = time when 63% of the maximum concentration (A_{max}) is attained
Each data value is the mean of 3 or 4 experiments ± standard error of the mean.

The A_{max} for P1 transport in the drug sensitive clone was 67.7 ± 9.7 pmoles/10^8 trypanosomes. This constitutes 48.1% of the total uptake. The rate constant for P1 was 60.4 ± 9.0 seconds and is comparable to the rate constants for the P2 transport (71.2 ± 9.7 seconds) and the overall uptake process (57.7 ± 10.3 seconds). This means that the P1 and P2 transporter molecules in the drug-sensitive trypanosomes operate at similar speed. The mean initial rate for P1 transport of 1.26 ± 0.2 pmoles/sec/ 10^8 trypanosomes was comparable to that of the P2 (1.63 ± 0.3 pmoles/sec/10^8 trypanosomes) in the same population.

In the drug-resistant clone, the A_{max} at the P1 transporter of 43.9 ± 2.0 pmoles/10^8 trypanosomes was slightly reduced (1.5-fold) compared to the wild type (T=2.4,

$P=0.14$, $N=3$). However the A_{max} appears greater than the A_{max} (33.6 ± 8.5 pmoles/10^8trypanosomes) at the P2 in the same parasites. The rate constant for the P1 (19.3 ± 5.8 seconds) was significantly reduced ($T=3.83$, $P=0.03$, $N=3$) indicating increased speed of activity. This rate constant was 2-fold lower than that of the P2 transporter (39.9 ± 9.0 seconds) in the same (resistant) trypanosomes. This means that the transporter molecules of the P1 are functioning at a faster speed than those of the P2 in the resistant trypanosomes. The initial rate for the P1 in the resistant clone was 2.68 ± 0.7 pmoles/sec/10^8 trypanosomes. This was 3.3 times higher than the initial rate obtained for the P2 transport, 1.5 times higher than that of the overall transport in the same trypanosomes and 2.1 times higher than the P1 of drug-sensitive trypanosomes. These differences were however, not statistically significant at 95% confidence level ($T=-1.86$; $P=0.2$; $N=3$.)

A summary illustration of observations on kinetic parameters of adenosine transport in drug sensitive and drug resistant *T. evansi* is shown in Figure 7.6.

Drug sensitive

Inosine
Adenosine:
A_{max} = 67.7
T = 60.4
Initial Rate = 1.26

P1

P2
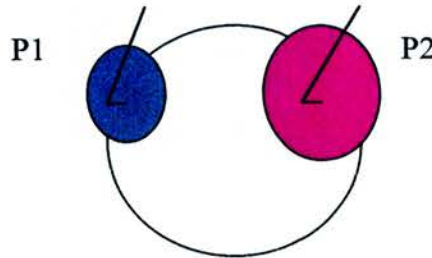
Mel Cy
Berenil
Adenine
Adenosine:
A_{max} = 112.5
T = 71.2
Initial Rate =1.63

Drug resistant

Inosine
Adenosine:
A_{max} = 43.9
T = 19.3
Initial Rate = 2.68

P1
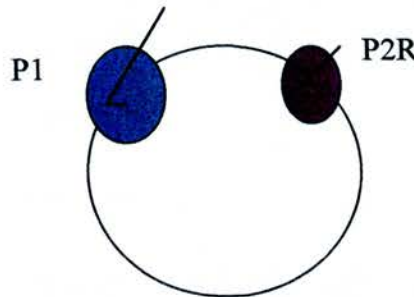
P2R Mel Cy
Berenil ?
Adenine
Adenosine:
A_{max} = 33.6
T = 39.0
Initial Rate =0.81

Figure 7.6: Summary illustrations of rate kinetic parameters of adenosine transport on P1 and P2 transporters in mel Cy sensitive and mel Cy resistant *T. evansi*. Details of experimental procedures are described in the text. The units of parameters are as follows: A_{max} (ρmole/10^8 trypanosomes); T (seconds); Initial Rate (ρmole /10^8 trypanosomes /second). Half arrows indicate transport.

7.3.2. Kinetics of adenosine uptake II : Effect of increasing concentration of substrate on uptake

The effect of increasing concentration of adenosine on velocity of uptake in the drug sensitive trypanosomes is shown in Figures 7.7A-D. A summary of the kinetic parameters estimated from these plots is given in Table 7.2.

The maximum velocity of uptake (U_{max}) for the overall uptake (in the absence of inhibitor) was 37.4 ± 5.4 pmol/10^8trypanosomes/5min. The mean K_m for this uptake was 0.38 ± 0.1 µM.

The mean U_{max} of the P1 transport in the WT parasites was 12.4 ± 2.3 pmol/10^8trypanosomes/5min. A low K_m for P1 transport (0.07 ± 0.03) µM was observed.

For the P2 transport the U_{max} was 28.9 ± 5.1 pmol/10^8 trypanosomes/5min., being over 2-fold higher than U_{max} for P1. The K_m of P2 transporter was 0.74 ± 0.3 µM which is about 10 times higher than the K_m for P1 transport.

Similar plots giving the effects of concentration of substrate on uptake of adenosine in drug-resistant clone are shown in Figure 7.8A-D. The summary of kinetic parameters estimated from the plots are included in Table 7.2.

The U_{max} for the overall uptake in the absence of inhibitor in these trypanosomes was reduced slightly (T=2.12, P=0.1, N=4) amounting to 22.2 ± 4.7 pmol/10^8 trypanosomes/5min. The K_m for this process was not significantly (T=1.66, P=0.24, N=4) reduced to 0.14 ± 0.03 µM compared to $0.38\pm$ µM in the drug sensitive population.

The U_{max} of P1 transport in the drug resistant clone was reduced about 2-fold, to 6.7 ± 1.8 pmol/10^8trypanosomes/5min. The difference was, however, not significant (T=1.98, P=0.12, N=4). The K_m for P1 of 0.06 ± 0.02 µM was similar (T=0.34, P=0.77, N=4) to the drug sensitive trypanosomes (0.07 ± 0.03 µM).

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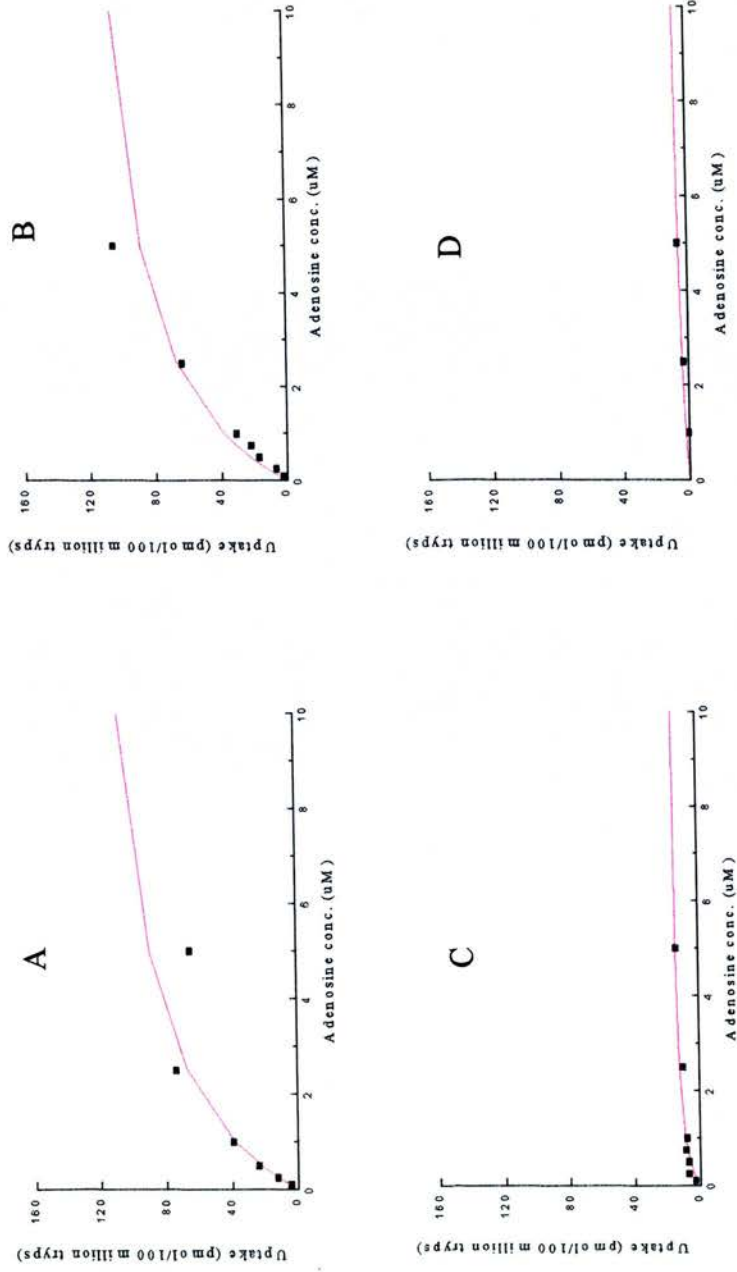
Figure 7.7: Transport of adenosine in drug sensitive *Trypanosoma evansi* (WT). II: Effect of increasing concentration of substrate on uptake. 1×10^7 trypanosomes were incubated with varying concentrations of tritiated adenosine, in the absence of inhibitor (A); or presence of 1 mM inosine (B), 1 mM adenine (C), or 1 mM of both adenine and inosine (D), for 5 minutes, at 37°C. Uptake was calculated from incorporated radioactivity and expressed as pmoles/10^8 trypanosomes. Data was fitted to rectangular hyperbolic plots and analysed using non-linear regression analysis programme. Kinetic parameters were determined from these plots using Michaelis-Menten equation. Each experiment was repeated 3 or 4 times and similar results obtained.
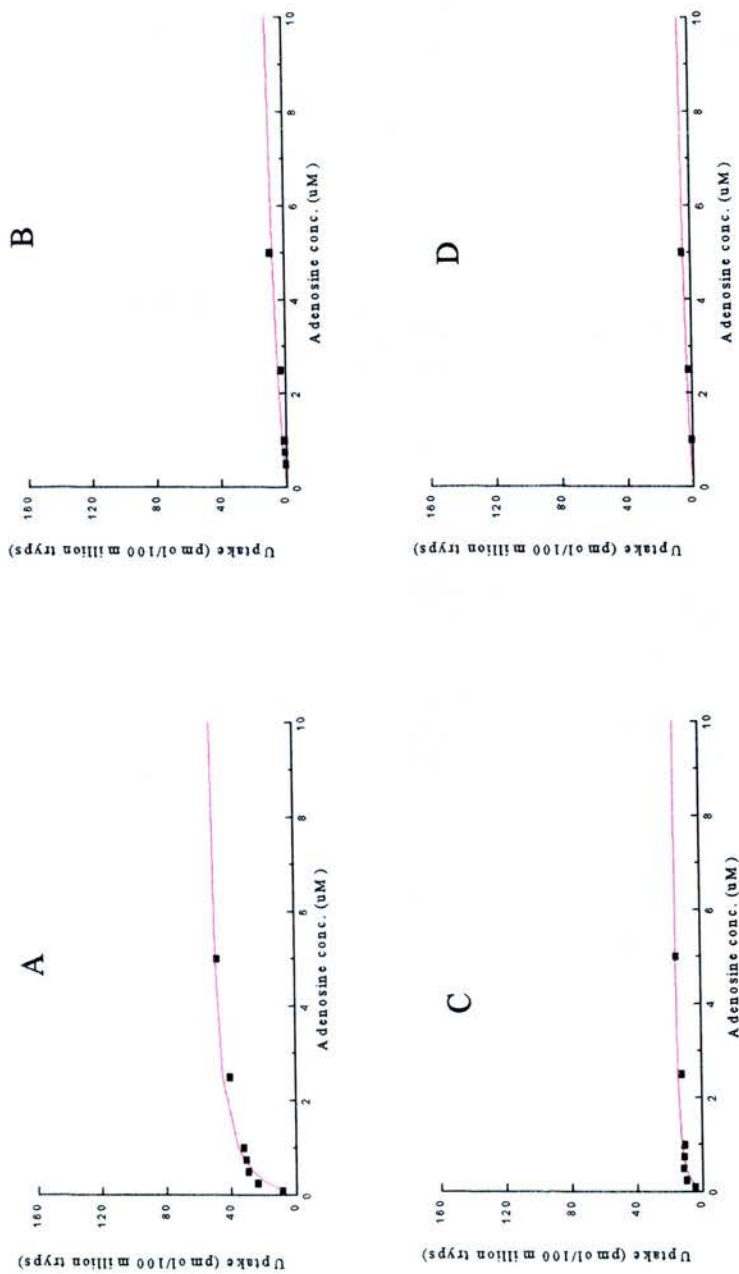
Figure 7.8: Transport of adenosine in drug resistant *Trypanosoma evansi* (CR3.1). II: Effect of increasing concentration of substrate on uptake. 1x 10^{7} trypanosomes were incubated with varying concentrations of tritiated adenosine, in the absence of inhibitor (A); or presence of 1 mM inosine (B), 1 mM adenine (C), or 1 mM of both adenine and inosine (D), for 5 minutes, at 37°C. Uptake was calculated from incorporated radioactivity and expressed as pmoles/10[8] trypanosomes. Kinetic parameters were determined from these plots and analysed using non-linear regression analysis programme. Each experiment was repeated 3 or 4 times and similar results were obtained.

The U_{max} of P2 transport in the drug resistant clone was significantly (T=4.89, P=0.039, N=4) reduced to 3.3±1.1 ρmol/10^8 trypanosomes/5min. This represents approximately 9-fold decrease compared to the susceptible clone. The K_m of 0.1±0.02 μM also represents 7-fold decrease compared to the wild type parasites. Statistical comparison did not show a significant difference (T=1.82, P=0.21, N=4).

Table 7.2: Kinetic parameters of adenosine transport in drug sensitive and drug resistant *Trypanosoma evansi*. II: Effect of substrate concentration on uptake.

Adenosine transport function	Drug sensitive (WT)		Drug resistant (CR3.1)	
	U_{max} (ρmol/10^8cells/ 5 min)	K_m (μM)	U_{max} (ρmol/10^8cells/ 5min)	K_m (μM)
Total uptake	37.4±5.4	0.38±0.1	22.2±4.7	0.14±0.03
Adenine present (P1)	12.4±2.3	0.07±0.03	6.7±1.8	0.06±0.02
Inosine present (P2)	28.9±5.1	0.74±0.3	3.3±1.1	0.1±0.02

Legend: U_{max} = maximum velocity of uptake. Represents the maximal rate at which a transporter functions. It is attained when the transporter is saturated with substrate. K_m = dissociation constant. The K_m value of a transporter indicates the concentration of substrate at which half the active sites are filled and represents the strength with which a transporter binds to its substrate (Cohn and Gottlieb, 1997).
Each data value is the mean of 3 or 4 experiments ± standard error of the mean.

7.3.3. Kinetics of inhibition of adenosine uptake in *Trypanosoma evansi*

The mean values for the dissociation constant (K_i) for the various inhibitors at the P1 or P2 transporters in the drug sensitive and drug resistant trypanosomes are shown in Table 7.3.

Inosine, adenine and Berenil® inhibited adenosine uptake on either the P1 or P2 with very low K_i values. In the drug sensitive trypanosomes the K_i for inosine and adenine alone were 0.002± 0.001μM and 0.28± 0.11μM, respectively. Little

interaction was observed with Berenil® as K_i values remained zero. The K_i for inosine on the P1 was 0.03±0.004 µM. The K_i for adenine and Berenil® on the P2 were 0.3±0.1 µM and 0.01±0.01 µM respectively. There was no interaction with Berenil® on the P1 transporter (K_i = 0.00 µM).

In the drug resistant trypanosomes the K_i for inosine on the P1 (0.063±0.03 µM) was slightly (2-fold) increased compared to the drug sensitive trypanosomes. However at the P2 the K_i for adenine had increased 6.5-fold (1.94±0.3 µM) compared to the drug sensitive trypanosomes. There was little or no interaction with Berenil® at the P2 in these trypanosomes (K_i = 0.00 µM)

The K_i for inosine was lower than that for adenine in both the drug sensitive (10-fold) and the drug resistant (30-times) trypanosomes.

Table 7.3: Kinetics of inhibition of adenosine transport in drug-sensitive and drug-resistant *Trypanosoma evansi*.

Inhibitor (Transporter)	K_i (µM)	
	Drug sensitive	Drug resistant
Inosine (P1)	0.03±0.004	0.063±0.03
Adenine (P2)	0.3±0.10	1.940±0.30
Berenil (P2)	0.01±0.01	0.00
Berenil (P1)	0.00	-
Inosine alone	0.002±0.001	-
Adenine alone	0.28±0.11	-
Berenil alone	0.00	-

Legend: Uptake of 1 µM adenosine by 10^7 trypanosomes was measured at 37°C, for 5 minutes. Increasing concentrations of inosine, adenine or Berenil were included in reaction medium. The effect of inhibition at the P1 or P2 were determined by saturation of the alternate transporter with either 1 mM inosine or 1 mM adenine. The concentrations of inosine used were 0.01, 0.1, 1, 10, 50 100, 1000 µM; adenine were 0.01, 0.1, 1, 10, 50, 100 µM; and Berenil were 0.01, 0.1, 1, 10, 5, 100 µM. Experiments were repeated 2-4 times and similar results were obtained.

- = Not done

A summary of observations on kinetic parameters of adenosine uptake in drug resistant *T. evansi* is shown in Figure 7.9.

An overall summary illustrating the main alterations in adenosine uptake in the drug resistant trypanosomes is shown in Figure 7.10.

Drug sensitive

Inosine: $K_i = 0.03$ P1
Adenosine:
$U_{max} = 12.4$
$K_m = 0.07$

P2 Mel Cy
Berenil: $K_i = 0.01$
Adenine: $K_i = 0.3$
Adenosine:
$U_{max} = 28.9$
$K_m = 0.74$

Drug resistant

Inosine: $K_i = 0.06$ P1
Adenosine:
$U_{max} = 6.7$
$K_m = 0.06$

P2R Mel Cy
Berenil: $K_i = 0.0$
Adenine: $K_i = 1.94$
Adenosine:
$U_{max} = 3.3$
$K_m = 0.1$
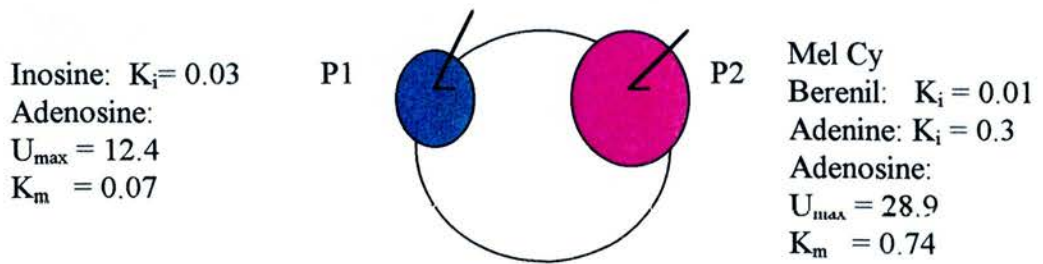
Figure 7.9: Summary illustrations of Michaelis-Menten kinetic parameters for adenosine uptake on the P1 and P2 transporters in mel Cy sensitive and mel Cy resistant *T. evansi*. Details of experimental procedures are described in the text. The units of parameters are as follows: U_{max} (ρmole/10^8 trypanosomes/5 minutes); K_m (μM); K_i (μM). Half arrows indicate uptake.
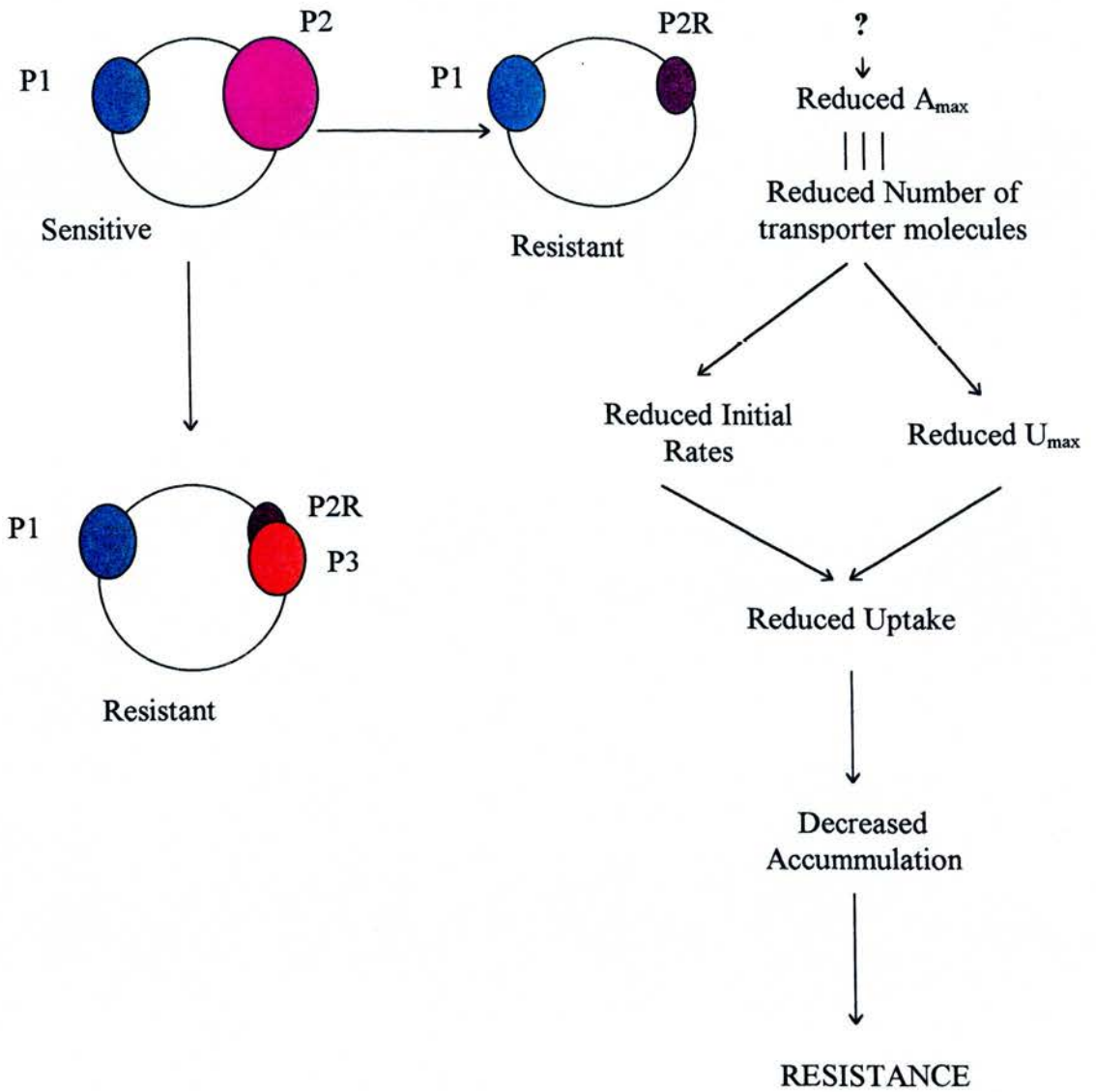
Figure 7.10: Summary illustrations of changes in kinetic parameters of adenosine transport in mel Cy resistant *T. evansi*.

7.4. DISCUSSION

Presence of P1/P2 bipartite adenosine transport system in *Trypanosoma evansi:*

Transport of 5 µM adenosine was followed between 10 and 120 seconds in a drug-sensitive *T. evansi* isolate and its mel Cy-resistant derivative clone, and the various kinetic parameters measured. The results confirm the observations from inhibition experiments (Chapter 6) that there are two adenosine transport systems in this strain of *T. evansi* (TREU 1840). The finding of bipartite adenosine transport systems in *T. evansi* is consistent with previous reports on *T. brucei* (Carter and Fairlamb, 1993) and *T. equiperdum* (Barrett, *et al.*, 1995). Ross and Barns (1996) however, reported observation of three adenosine transporters in another strain of *T. evansi* (TREU 1981). It is possible that different strains of *T. evansi* differ in their adenosine transport systems. However, the report of Ross and Barns (1996) did not include investigations on the kinetic properties of these transporters. Conclusions were based on sequential inhibition of adenosine uptake with inosine, adenine and Berenil®. This, though indicative, may not have provided sufficient information or conclusive evidence on the number of transporters present.

Reduced uptake rates at the P2 in drug resistant *Trypanosoma evansi*:

It was found that the maximum concentration or saturation point (A_{max}) for the P2 transport was reduced (3.4-fold) in the drug-resistant trypanosomes. On the other hand, the Rate constant for the same transporter was also reduced, suggesting that individual transporter molecules were operating at a faster speed. However, the initial rate of uptake at the P2 was reduced 2-fold. These findings suggest that the reduced uptake capacity (A_{max}) together with reduced initial rates of transport are responsible for the reduced uptake at the P2 transporter in drug-resistant trypanosomes. Since A_{max} is the maximum uptake at infinite time, it may be considered to represent accumulation of substrate in the parasite. Reduced A_{max} therefore, represents reduced potential for accumulation of the substrate intracellularly in drug resistant trypanosomes.

The lysis as well as adenosine uptake inhibition studies presented here (see Chapters 5 and 6) show that mel Cy competes with adenosine (and adenine) on the P2 transporter in *T. evansi*. Hence reduced substrate accumulation at the P2 suggests that resistance to mel Cy is a result of reduced accumulation of the drug. Drug resistance as a result of reduced accumulation has been reported in trypanosomes with stilbamidine (Fulton and Grant, 1955), pentamidine (Damper and Patton, 1976; Carter *et al.*, 1996), and, isometamidium (Sutherland *et al.*, 1991, 1992a). It has also been shown in multidrug-resistant trypanosomes (Frommel and Balber, 1987); and in other parasites such as *Plasmodium falciparum* (Krogstad *et al.*, 1987), and *Entamoeba histolytica* (Samuelson *et al.*, 1990).

The uptake capacity (A_{max}) of a transporter is a function of: i) the number (concentration) of the transporter molecules available; ii) the speed of operation of individual transporter molecules (represented by the rate constant); and, iii) the concentration of substrate. Under the experimental conditions used in this study 5 μM concentration of adenosine was sufficient to provide saturable conditions for maximum uptake by trypanosomes. Thus variability due to insufficient substrate concentration was eliminated. Similarly, a lower rate constant obtained for the P2 transporter in the drug resistant trypanosomes is indicative of increased speed. Thus the reduction in uptake is not a result of slow activity of the individual transporter molecules. Reduced number of available transporter molecules could only account for the reduction in A_{max} and consequently initial rate of transport at the P2.

Non-specific selection effect of mel Cy:

It is generally considered that drugs are not 100% specific for their target molecules (Martin R.J., personal communication). High concentrations or dose levels may possibly have some non-specific selective effect for the P1 in addition to the P2 transporters. This may account for the slightly reduced A_{max} at the P1 transporter in the drug resistant trypanosomes. Observations of slight reductions in adenosine transport by P1 transporter have also been reported in *T. brucei* selected using sodium melarsen (Carter and Fairlamb, 1993), and *T. equiperdum* using Berenil® (Barrett *et al.*, 1995). That the rate constant for P1 was also reduced in the resistant

trypanosomes in this study further supports the possibility of non-specific selection effects by the drug.

It was also observed that the sum of the A_{max} values for the P1 and P2 processes in the unselected clone was greater than the mean of the overall transport in the absence of inhibitor. This was however, not the case in the resistant trypanosomes. In the latter the sum of the A_{max} of the two individual transporters, P1 and P2, was less than the overall uptake. It is possible that the two transport processes, P1 and P2, are not entirely independent. There may be an overlap in which case the apparent capacity of one process is a combination of the capacity of that process plus an additional component which is common between the two. In this situation the sum total of the two processes measured individually would be more than the overall uptake. In the mel Cy-resistant trypanosomes, however, one of the two processes (P2) is greatly deficient. This may have disturbed the balance in the relationship between the two transporters such that the sum of the two, measured independently, did not amount to the overall process.

The increased speed of activity of both the P1 and P2 transporter molecules in drug resistant *T. evansi* could be a compensatory switch mechanism by the trypanosomes to make up for the reduced uptake at the P2 transporter.

Effect of substrate concentration on uptake:

Michaelis-Menten kinetics of adenosine uptake were determined in both the drug sensitive and drug resistant trypanosomes. The results also confirmed the presence of two adenosine transporters in *T. evansi* TREU 1840, consistent with reports on other Trypanosomatidae (James and Born, 1980; Okochi *et al.*, 1983; Baer *et al.*, 1992; Carter and Fairlamb, 1993; Barrett *et al.* 1995).

In the drug-sensitive clone the U_{max} for P2 transport was over 2 times higher than that for the P1 transport. This compared well with the Rates parameters in the same parasites. The U_{max} for P1 (12.4 pmol/10^8trypanosomes/5min) observed for *T. evansi* is comparable to the V_{max} (10.6 pmol/10^8trypanosomes/sec) reported for *T. brucei* (Carter and Fairlamb, 1993) and *T. equiperdum* (8.4 pmol/10^8trypanosomes/sec) (Barrett *et al.*, 1995). The U_{max} for P2 (28.9 pmol/10^8trypanosomes/5min.) in *T.*

evansi in this study appears higher than reported values for *T. brucei* (9.5 pmol/10^8 trypanosomes/sec) and *T. equiperdum* (6.9 pmol/10^8trypanosomes/sec). Carter *et al.* (1996) also reported transport of pentamidine on the P2 transporter in *T. brucei* with a V_{max} of 9.35 pmol s^{-1} (10^8 cells)$^{-1}$.

The K_m for P1 transport (0.07 µM) obtained for *T. evansi* was similar to that reported for the P1 in *T. brucei* (0.15 uM) (Carter and Fairlamb, 1993), although a relatively higher value (0.60 µM) was observed for *T. equiperdum* (Barrett *et al.*, 1995). The K_m for P2 (0.74 µM) in *T. evansi* determined in this study was also very similar to those reported for *T. brucei* (0.59 µM) and for *T. equiperdum* (0.7 µM). A K_m of 0.84 µM was reported for pentamidine on this transporter (Carter *et al.*, 1996).

This study measured uptake at 37°C in contrast to previous observations at 25°C (e.g. Carter and Fairlamb, 1993; Barrett *et al.*, 1995). A temperature of 37°C approximates the body temperature and represents the natural environment under which the *T. evansi* bloodstream forms live in their host. This provided a more ideal physiological atmosphere for uptake to take place.

The relatively high U_{max} for the P2 coupled with higher A_{max} at the same transporter supports the observation in this study that P2 transporter is a larger transport process compared to the P1 in drug sensitive *T. evansi*. In *T. brucei* and *T. equiperdum* the P2 was the smaller component of the two transporters. This confirms the variability in adenosine transport functions in different species of trypanosomes.

The longer incubation period employed in this study may have enhanced passive diffusion of adenosine into trypanosomes. This was however, corrected for using the modified Michaelis-Menten equation as described in the methods. It was not necessary to correct for passive diffusion component in the data obtained from time course experiments in view of the short incubation times used. Exponential plots obtained from this data did not indicate the presence of passive diffusion. Thus the kinetic parameters presented here are accurate measures of the carrier-mediated uptake processes at the P1 and P2 in *T. evansi* TREU 1840.

Alterations observed in the maximum velocity of uptake (U_{max}) in the drug resistant clone were similar to those observed in the corresponding A_{max} in these parasites.

While the P1 and P2 adenosine transporters have been previously characterised in drug sensitive trypanosomes (Carter and Fairlamb, 1993, Barrett *et al.*, 1995) very little information has been reported on the detailed kinetic changes in the P2 in drug resistant trypanosomes. Carter and Fairlamb (1993) observed that there was no detectable rate of transport of 1 μM adenosine on the P2 in a melarsen-resistant *T. brucei* clone. On this basis it was concluded that P2 transport was absent in this clone. Barrett *et al.* (1995) also determined the K_m (0.66 μM) and V_{max} (4.15) for the P1 transporter in Berenil®-resistant *T. equiperdum.*

This study show that a P2 transport process is still present in mel Cy resistant *T. evansi* although markedly reduced. Barrett *et al.* (1995) also reported the presence of a P2 component in Berenil®-resistant *T. equiperdum.* However these authors were not able to determine detailed kinetic parameters for the P2 transporter in the resistant clone.

This study has shown for the first time a detailed characterisation of the changes in kinetic parameters occurring at the P2 adenosine transporter in arsenical resistant trypanosomes.

Arsenical resistant *T. evansi* maintained high affinity for substrate on the P2 transporter:

The report of Barrett *et al.* (1995) suggested that loss of affinity for substrate was responsible for the reduced uptake by the P2 transporter. This conclusion, however, was not based on kinetic data but on inhibition studies as well as response to drug. From the present study the K_m of the P2 in mel Cy-resistant trypanosomes was not increased as expected but rather decreased 7-fold. By definition dissociation constant (K_m) is the reciprocal of affinity of binding between substrate and the transporter. A decreased K_m means that there is increased affinity for substrate at the P2 transporter in the drug-resistant clone. Thus the reduced uptake at the P2 transporter observed in this study was not a result of reduced affinity.

Reduced numbers of transporter molecules is responsible for reduced uptake at the P2 in arsenical resistant *Trypanosoma evansi*:

Besides the Michaelis-Menten kinetics this study also measured other kinetic parameters for the P1 and P2 transporters, and the overall uptake. The two parameters, A_{max} and the rate constant (T), have not been previously determined for the P1 and P2 adenosine transporters in trypanosomes. These rate parameters complimented results obtained from Michaelis-Menten kinetics in both the drug sensitive and resistant trypanosomes.

The rate constant determined for the P2 show that the speed of the P2 transporter molecules did not decrease. (Indeed the speed of both the P1 and P2 transporter molecules had increased in the drug-resistant trypanosomes). However the initial rate for the P2 had decreased in this clone. The significant change in A_{max} at the P2 suggests that the decrease in initial rate of transport was a result of decrease in A_{max}.

It is concluded that reduced uptake capacity (A_{max}) at the P2 transporter is responsible for the reduced initial rate of transport and reduced velocity (U_{max}). By definition A_{max} represents the maximum intracellular concentration (uptake) of substrate at time infinity. It is considered that in the presence of saturating concentrations of substrate (and at time infinity) as used in this study then, A_{max} is directly a function of the concentration or numbers of transporter molecules available. Thus reduced A_{max} signifies reduced concentration or numbers of transporter molecules. All of these would result to the reduced uptake observed on this transporter. These factors are also likely to result in reduced overall uptake and possibly reduced accumulation of substrate in the drug resistant parasites.

The marked change in the K_m for the P2 together with changes in A_{max} and U_{max} in the resistant clone suggests that the P2 transporter has mutated to what is now described as a P2R transporter. The P2R transporter possibly interacts with Berenil® in a different way. It is also possible that a completely new transporter is being produced in place of the P2 in the resistant trypanosomes.

It is not known what specific mutations are responsible for the reduction in numbers of the P2 transporter molecules in arsenical-resistant trypanosomes.

Kinetics of inhibition of adenosine uptake on the P1 and P2 transporters:

Low K_i values for inhibition of adenosine uptake were obtained for both inosine and adenine from this study. The K_i for adenine on the P2 of 0.3 μM was very similar to that reported for adenine on the same transporter in *T. brucei* (Carter and Fairlamb, 1993). K_i values for inhibition by inosine or Berenil[®] have not been previously reported, although pentamidine has been shown to strongly inhibit adenosine uptake on the P2 with K_i of 0.56 μM (Carter *et al.*, 1996).

This study showed a consistently lower K_i for inosine on the P1 compared to the K_i for adenine on the P2. This indicates that in drug sensitive trypanosomes the P1 transporter binds to inosine with greater affinity than does the P2 for adenine. Based on affinity alone it would be expected that inosine will be a more potent inhibitor of adenosine uptake than adenine. This is further supported by the observation that relative to adenosine the K_i for inosine on the P1 and adenine on the P2 were equally lower (approximately 2-fold) than the K_m for adenosine on either transporter. That adenine rather than inosine was a more potent inhibitor of adenosine uptake supports the present observation that maximum uptake capacity (A_{max}) accounted for increased uptake on the P2 in drug sensitive trypanosomes. In the drug resistant trypanosomes the A_{max} on the P2 had greatly reduced resulting to decreased uptake.

A different picture was found in drug resistant trypanosomes. The K_i for inosine on P1 remained similar to that observed in the drug sensitive trypanosomes. (Similarly the K_m for adenosine on this transporter was not changed in these trypanosomes.) However the K_i for adenine on P2 increased 6.5-fold, indicating a marked decrease in affinity for adenine. The K_m for adenosine on this transporter had however, decreased 7-fold compared to drug sensitive trypanosomes.

Presence of a new adenosine transporter, P3, in arsenical resistant *Trypanosoma evansi*:

The major changes in K_i for adenine, K_m for adenosine, together with the changes in A_{max}, rate constant, U_{max} and the initial rate, on the P2, provide sufficient evidence that the transporter in the resistant trypanosomes is completely different from the P2 in the drug sensitive trypanosomes.

It is concluded that a new transporter, which is termed here P3, is present in arsenical resistant *T. evansi*. The P3 may have been selected during induction of resistance to mel Cy or may be a replacement for the P2 by the trypanosomes. It is logical to expect that a remnant of the P2 and the new, P3 transporter, may coexist in arsenical resistant trypanosomes.

The presence of a third transporter explains the apparently high overall uptake in the drug resistant clone inspite of the marked decrease in P2 activity. This switch to P3 represents another important adaptive mechanism by which drug resistant trypanosomes maintain their purine needs in the face of interferences with already existing pathways.

CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSIONS

Aims and experimental design:

The aim of this study was to investigate the biochemical basis for the development of resistance to arsenical drugs in *T. evansi*, and to determine if the mode of resistance development *in vitro* was similar to that developed in animals. The experimental design involved production and, characterisation of cloned lines of *T. evansi in vivo* and *in vitro* with varying degrees of sensitivity to mel Cy.

The resistance induced using mel Cy was characterised with respect to stability of resistance and cross resistance to other trypanocides. The mode of uptake of melaminophenyl arsenicals represented by mel Cy and mel B into *T. evansi* was investigated using an *in vitro* lysis assay. A detailed investigation, including a kinetic study of purine (adenosine) transport pathways of both drug sensitive and drug resistant trypanosomes was undertaken. Alterations in transport associated with the expression of the resistant phenotype were determined from these studies.

Influence of host immunity on resistance development:

The results showed that high levels of drug resistance can be easily produced in immunosuppressed hosts as well as in *in vitro* systems, but not in immunocompetent hosts. This observation highlights the prominent role of the host environment, specifically host immunity in determining the efficacy of trypanocidal drugs and the development of drug resistance. This phenomenon has also been observed in the chemotherapy of other parasitic infections (Doenhoff *et al.*, 1991).

The importance of the involvement of host immunity in chemotherapy of trypanosomosis is best appreciated when it is considered that animals that come under natural challenge with trypanosomes in endemic areas are constantly prone to various disease or stress conditions which impose a state of immunosuppression on

these animals. This study showed that such animals would provide a medium for the selection of drug resistant trypanosomes. This could be an important factor contributing to the widespread occurrence of resistance to trypanocides on the field.

It is suggested that in attempting to control trypanosomosis efforts should be directed not only at the trypanosome parasite alone but also on other concurrent parasitic infections prevalent in affected animals within that locality. This calls for integrated approach to the control of trypanosomosis requiring complete herd health programmes rather than individual disease control. This thought is recently being shared by other workers (see Holmes, 1997).

The fact that drug resistant trypanosomes produced in this study maintained their natural characteristics, including infectivity in the host, is important in the maintenance of resistant parasites under field conditions. This increases the risk of a wide scale spread of resistance once induced. Furthermore, a reduction in virulence of drug resistant trypanosomes such as observed in this study means a host harbouring resistant trypanosomes is likely to live longer than one with drug sensitive parasites (assuming no mixed resistant/sensitive infections occurred). This shows a trend towards adaptation between the resistant parasite and the host which favours the survival of the parasite and consequently maintenance of resistance in nature. On the other hand in a situation of mixed drug sensitive/ resistant infections, if reduced virulence was a factor of reduced growth rate of the drug resistant trypanosomes as has been suggested (Mutugi *et al.*, 1996), it might be expected that with time the drug sensitive populations may outgrow the resistant one. The resistant population may then decline after several generations of transmission from hosts to host. Detailed study of the dynamics of transmission and maintenance of drug resistant parasites is needed.

Absence of naturally resistant sub-populations of *Trypanosoma evansi* isolate:

The development of resistance to mel Cy in *T. evansi* populations was a result of induced mutation and subsequent selection of resistant mutants. Experiments failed to demonstrate the existence of high level resistant mutants either *in vivo* or *in vitro*. However, this does not rule out completely the possibility of occurrence of some

mutants with very low resistance prior to induction. These probably, could not survive the selective effect of the drug at the concentrations tested.

Cross resistance between mel Cy and other trypanocides:

Resistance induced against mel Cy conferred a high degree of cross-resistance to mel B, another melaminophenyl arsenical drug, and to Berenil®, a diamidine. In circumstances where widespread resistance to a drug occurs there is usually a need to introduce an alternative drug to which trypanosomes respond. In such a situation this study suggests that introduction of mel Cy would alleviate the problem of resistance to suramin and to a lesser extent quinapyramine. Both of these are drugs that have been commonly used in the control of trypanosomosis caused by *T. evansi*.

Mel Cy has been reported to be effective against diminazene aceturate-resistant *T. brucei* (Zweygarth and Kaminsky, 1990). Field isolates of *T. evansi* and *T. equiperdum* that were resistant to Berenil®, suramin, quinapyramine or isometamidium were also reported to have remained fully sensitive to mel Cy. This led to the suggestion that mel Cy might be an effective trypanocide for controlling veterinary trypanosomosis (Zhang *et al.*, 1992). It has however, been shown (Zhang *et al.*, 1993) that trypanosomes selected for resistance using Berenil® exhibited two mechanisms of resistance - one expressing cross resistance to mel Cy and the other showing no cross resistance to mel Cy. In the light of this report and the cross resistance between mel Cy and Berenil® observed in the present work, it is envisaged that mel Cy can only be used to a limited extent as a substitute in situations where Berenil® resistance occurs. A switch to Berenil® may also not be useful where mel Cy resistant trypanosomes are prevalent. Similarly, these results underscore the potential problems that might arise in any attempts to use mel Cy and Berenil® concurrently in an endemic area to control trypanosomosis as this will only increase the chances of development of resistance to both drugs.

Differences in uptake mechanisms between melaminophenyl arsenical drugs:

Mel Cy and mel B, both trivalent melaminophenyl arsenicals caused *in vitro* lysis of *T. evansi* in a manner that indicated that specific transport mechanisms were involved

in the uptake of these drugs into trypanosomes. However, attempts at inhibition of lysis by these drugs using purine compounds revealed that there were differences in the mode of uptake of the two arsenicals. The study showed that mel Cy was taken up by an adenine/adenosine transporter, similar to *T. brucei* (Carter and Fairlamb, 1993). Mel B was not recognised by either of the two adenosine transporters. The blocking of both mel Cy and mel B lysis by Berenil® further raises questions on the specific mechanisms of uptake of mel B and the diamidines. The basis for the cross resistance between mel Cy and mel B observed in this and previous studies also warrants further investigation.

A bipartite adenosine transport system maintained in arsenical resistant *Trypanosoma evansi*:

Studies on adenosine uptake in *T. evansi* showed that these parasites possess a P1/P2 bipartite adenosine transport system similar to *T. brucei* (Carter and Fairlamb, 1993) and *T. equiperdum* (Barrett *et al.*, 1995), and mel Cy interacted with the P2 transporter.

The P2 transporter was maintained in arsenical resistant *T. evansi*. However the activity of the P2 transporter was modified resulting to reduced uptake at the transporter, and a reduction in total adenosine uptake by the parasite. Observations of reduction in activity of the P2 transporter were previously reported in *T. equiperdum* (Barrett *et al.* (1995). However, in contrast to suggestions by Barrett *et al* (1995) this study showed that reduced uptake at the P2 in arsenical resistant *T. evansi* was not due to reduced affinity for substrate. The drug resistant trypanosomes actually showed increased affinity for substrate indicated by a 7-fold decrease in K_m at the P2 compared to drug sensitive parasites.

Reduced numbers of transporter molecules is responsible for reduced uptake at the P2 transporter:

A detailed kinetic study of the P1 and P2 transporters showed that reduced uptake at the P2 was a result of reduced maximum capacity (A_{max}) of the transporter, reduced initial transport rate, and reduced initial velocity of uptake. The results also showed increases in speed of activity of both the P1 and P2 transporter molecules in drug

resistant trypanosomes. From the kinetic parameters determined it was found that reduced A_{max} was the major factor that possibly gave rise to other changes observed at the P2. A_{max} was shown to be an index of the numbers or concentrations of transporter molecules available. Hence reduction in A_{max} at the P2 represents a reduction in the concentration of transporter molecules. This is likely to have resulted in reduced initial transport rates and reduced initial velocity of uptake observed. Consequently, these may have resulted in decreased uptake and finally decreased intracellular accumulation of substrate in drug resistant trypanosomes.

If the P2 transporter is still present, but not capable of concentrating substrate as rapidly as in the drug sensitive trypanosomes it means that it would require only a longer time (and probably higher concentrations) for toxic substrates such as mel Cy to be accumulated at concentrations high enough to cause cytotoxicity in drug resistant trypanosomes. However beyond that time or concentration level the resistant organisms would become susceptible to the effect of the drug. This may explain the pattern of the *in vitro* lysis by mel Cy observed in the drug resistant trypanosomes. These were partially lysed by relatively low concentrations (10-50 µM) but at the same time were resistant to the dose (1 µM) that caused rapid lysis of drug sensitive parasites. It is logical to expect that the more the decrease in P2 activity the higher the dose required to cause lysis of the resistant trypanosomes, which is what was observed in this study. It is considered that under *in vivo* conditions, and in drugs which are rapidly excreted from the body such as mel Cy (Raynaud *et al*, 1989b) decreased intracellular accumulation as a result of decreased uptake could provide a double advantage to the drug resistant trypanosomes.

Cross resistance between mel Cy and Berenil is a result of common mechanism of uptake:

This study showed that Berenil® interacted with the P2 transporter, in similar manner to mel Cy, in drug sensitive *T. evansi*. This provides evidence that the cross resistance observed between mel Cy and Berenil® is due to a common mechanism of uptake. However, the fact that mel Cy and mel B have been shown in this study to have different modes of uptake and that lysis by both drugs was blocked by Berenil®

invites speculation on other possible mechanisms involved in transport of melaminophenyl arsenicals and the diamidines. Obviously the proposition of the existence of one transporter that mediates for all melaminophenyl arsenicals and the diamidines, based on the findings of this study, is not tenable. Alternative modes of entry for arsenicals such as mel B, and possibly Berenil® may exist which have not yet been identified.

Alterations in P2 transporter of drug resistant *Trypanosoma evansi*:

The marked change in the K_m of the P2 transporter, besides other changes such as A_{max}, U_{max} and initial transport rates, in drug resistant trypanosomes provides strong evidence that the P2 transporter has mutated and possibly functions as a different transporter. We termed this transporter P2R in this study. This was evident from the changes in the interaction with Berenil® both in the presence of inosine and adenine in these trypanosomes.

Kinetics of inhibition of adenosine uptake:

The kinetics of inhibition of adenosine uptake showed that inosine, adenine and Berenil® inhibited uptake on either the P1 or P2 transporters with very low K_i values. There was no interaction with Berenil® on the P1. In the drug resistant line the K_i for inosine on P1 was similar to that of the drug sensitive line. However, a 7-fold increase in K_i for adenine was observed on the P2.

A new adenosine transporter, P3, is present in arsenical resistant *Trypanosoma evansi*:

Based on changes in all the kinetic parameters of what should be the P2 it was concluded that a new transporter (P3) was present in the arsenical resistant trypanosomes. This P3 transporter may have been selected for during the induction of resistance to mel Cy, or may be an adaptation by the resistant trypanosomes following gross reductions in activity of the P2 transporter.

In conclusion this study has shown that the dynamics of interaction of substrate with the adenosine transporters in trypanosomes is very complex and may not be fully explained on the basis of competitive inhibitions and kinetic data alone. Molecular

studies involving isolation and cloning of the P2 and P3 transporters may fully elucidate the specific mutations responsible for arsenical resistance in these trypanosomes. It is also of interest to study the interaction of the P3 transporter with other alternative substrates, including toxic adenosine analogues. This may provide useful information for improved chemotherapy of trypanosomes resistant to arsenical compounds.

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Room A
Afternoon Session

3.20   3.40
2A17   2A18

In Vivo And *In Vitro* Selection Of Drug Resistant *Trypanosoma evansi* Isolates And A Comparison Of Their Drug Resistance Profiles

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Trypanosoma evansi bloodstream forms resistant to the arsenical trypanocide Melarsamine hydrochloride (Mel Cy, Cymelarsan) were derived from a drug sensitive isolate after induction in irradiated or cyclophosphamide-treated mice. The isolate was also adapted to axenic growth *in vitro* and drug resistant parasites were obtained by cultivation in the continuous presence of increasing concentrations of Mel Cy. Attempts were made to induce resistance in immunocompetent (normal) mice. Drug resistant trypanosomes were tested for their sensitivity *in vivo* to Mel Cy and compared with wild type parasites. Mice infected with wild type parasites were cured with 1.0 mg/kg Mel Cy. In contrast, all drug resistant parasite populations were not cured by 40.0 mg/kg, which is the highest tolerated dose in these animals. *In vitro* assays were also carried out to determine the sensitivity of the different trypanosome populations to Mel Cy and to other trypanocides. Cloned trypanosomes, derived after the induction of resistance to Mel Cy in cyclophosphamide-treated mice, exhibited greater tolerance to the drug than the uncloned parent populations. Cross resistance to other trypanocides was detected.