https://theses.gla.ac.uk/

Theses Digitisation:

https://www.gla.ac.uk/myglasgow/research/enlighten/theses/digitisation/

This is a digitised version of the original print thesis.

Genetic Analysis of Drug Resistance in

Trypanosoma brucei

UNIVERSITY
of
GLASGOW

A thesis submitted to the University of Glasgow for the Degree of

DOCTOR OF PHILOSOPHY

IN

INFECTION AND IMMUNITY

BY

SONYA DOROTHY ANNE TAYLOR

Division of Infection and Immunity
University of Glasgow
Glasgow, G12 8QQ
Scotland

February 2004

ProQuest Number: 10390820

ProQuest.

ProQuest 10390820

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

DECLARATION

I hereby declare that this thesis embodied the results of my own work (except where explicitly stated), that it has been composed by myself and that it does not include work forming part of another thesis presented successfully for a degree in this or another University.

Date   Signature

ABSTRACT

Genetic mapping, positional cloning and reverse genetics provide an alternative to the biochemical and molecular approaches used to date, to determine the basis of arsenical resistance in *Trypanosoma brucei*. Genetic mapping of loci determining phenotypes of relevance to diseases has proved to be a powerful approach in a number of organisms including humans and *Plasmodium falciparum*, particularly when coupled with a full genome sequence. In this thesis, this approach has been established in *T. brucei* by determining the genetic basis of naturally occurring arsenical resistance and undertaking linkage analysis using our recently developed genetic map.

I adapted a simple, verified screening assay for assessing drug sensitivity based on the use of AlamarBlue. Three stocks used as parents in genetic crosses differed in drug sensitivity: STIB 247–Sensitive, STIB 386–Resistant and TREU 927–Resistant. Genetic linkage analysis using 101 polymorphic markers identified from the extensive sequence available from the genome project was then used to examine inheritance of the drug resistance phenotype in *T. brucei*. From this, the co-segregation (into F1 progeny) of markers and the resistance phenotype was determined using crosses, 247 x 386 and 247 x 927. Inheritance of resistance in both crosses was compatible with a simple single locus genetic model with one dominant allele determining resistance. Linkage analysis showed that the locus conferring resistance lay within an ~25 kilobase region on Chromosome II, which contains 6 open reading frames (ORFs).

A reverse genetic approach was then used to disrupt alleles for each of the six ORFs in turn. An allele of one gene, Tb927.2.2380, was shown to determine resistance and this was confirmed by transfecting the resistance allele into a drug sensitive stock to generate an arsenical resistant line. This gene also determines cross-resistance to the major veterinary trypanocide, diminazene aceturate and has been named the arsenical and diamidine (ard) resistance gene.

ACKNOWLEDGEMENTS

Table of Contents

Chapter 1   General Introduction

List of Figures

List of Tables

Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
BBB	blood brain barrier
bp	base pair
BSA	bovine serum albumin
CBSS	Carter's balanced salt solution
cM	centimorgan
CNS	central Nervous System
CSF	cerebrospinal fluid
dddH$_2$O	double distilled de-ionised water
DFMO	Difluoromethyl ornithine
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DRD	dopamine receptor D
EDTA	Ethylene diamine tetra-acetate
G3PDH	Glycerol-3-phosphate dehydrogenase
G418	Geneticin
GPS	guinea pig serum
HAPT1	high affinity pentamidine transporter
Ig	immunoglobulin
I.p	Intra-peritoneal
kb	kilobase
LAPT1	low affinity pentamidine transporter

LDL	low density lipoprotein
LOD	log odds of difference
MBSU	Molecular Biology Sequencing Unit
MelB	Melarsoprol
MelCy	Cymelarsan
MelOx	Melarsen oxide
MelW	Trimelarsen
MIC	minimum curative dose
mRNA	messenger RNA
mM	milli-Molar
NIX	nucleotide identifier X
OD	optical density
ODC	ornithine decarboxylase
ORF	open reading frame
PBS	phosphate buffer saline
PBSG	phosphate buffer saline + 1% glucose
PCL	procyclic clones
PCR	polymerase chain reaction
PhenOx	Phenylarsine oxide
PIX	protein identifier X
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
SNP	single nucleotide polymorphism
STIB	Swiss Tropical Institute, Basel

Taq	*Thermus aquaticus*
TbAT1	*T. brucei* adenosine transporter 1
Tm	transmembrane domain
TREU	Trypanosome Research, Edinburgh University
UTR	untranslated region
VAT	variant antigen type
VSG	variant surface glycoprotein
WHO	World Health Organisation

Publications

Hall, N., Berriman, M., Lennard, N.J., Harris, B.R., Hertz-Fowler, C., Bart-Delabesse, E.N., Gerrard, C.S., Atkin, R.J., Barron, A.J., Bowman, S., Bray-Allen, S.P., Bringaud, F., Clark, L.N., Corton, C.H., Cronin, A., Davies, R., Doggett, J., Fraser, A., Grüter, E., Hall,S., Harper, A.D., . Kay, M.P., Leech, V., Mayes, R., Price, C., Quail, M.A., Rabbinowitsch, E., Reitter, C., Rutherford, K., Sasse, J., Sharp, S., Shownkeen, R., MacLeod, A., Taylor, S., Tweedie, A., Turner, C.M.R., Tait, A., Gull, K., Barrell, B. and Melville, S.E. (2003). The DNA sequence of chromosome 1 of an African trypanosome: gene content, chromosome organisation, recombination and polymorphism. *Nucleic Acids Research* **31**, 4864-4873.

El-Sayed, N.M.A., Ghedin, E., Song, J., MacLeod, A., Bringaud, F., Larkin, C., Wanless, D., Peterson, J., Hou, L., Taylor, S., Tweedie, A., Biteau, N., Khalak, H.G., Lin, X., Mason, T., Hannick, L., Caler, E., Blandin, G., Bartholomeu, D., Simpson, A.J., Kaul, S., Zhao, H., Pai, G., Van Aken, S., Utterback, T., Haas, B., Koo, H.L., Umayam, L., Suh, B., Gerrard, C., Leech, V., Qi, R., Zhou, S., Schwartz, D., Feldblyum, T., Salzberg, S., Tait, A., Turner, C.M.R., Ullu, E., White, O., Melville, S., Adams, M.D., Fraser, C.M. and Donelson, J.E. (2003). The sequence and analysis of *Trypanosoma brucei* chromosome II. *Nucleic Acids Research* **31**, 4856-4863.

CHAPTER 1

GENERAL INTRODUCTION

1.1 TRYPANOSOMES

The family Trypanosomatidae contain important pathogens of humans and domestic animals. The diseases caused by these protozoa are endemic or enzootic in different areas of the world and are responsible for serious medical and economic problems. Trypanosomatids are unicellular obligate parasites found in a wide variety of invertebrate and vertebrate hosts in nature. A number of these flagellates have monogenetic (one host) life cycles, but most of the notorious members of the family are the digenetic (two host) species responsible for the vector borne diseases including leishmaniasis and trypanosomiasis, which affect humans and domestic livestock in the tropical and sub-tropical areas of the world (Gibson and Stevens, 1999).

There are many species of *Trypanosoma* that live in their host without causing any disease, however there are a number of species that represent a disease complex, commonly referred to as trypanosomiasis. Those which cause most concern to man are the tsetse transmitted trypanosomes of Sub-Saharan Africa and the reduviid transmitted trypanosomes of South America. Chagas' disease caused by *Trypanosoma cruzi* occurs throughout Latin America and in terms of human infectivity it is by far the most important species, causing disease in approximately 20 million people annually in Central and South America. In contrast, African trypanosome infection in humans is markedly lower. Although there is an estimated 60 million people living in 300 disease

foci in Africa that are at risk to infection, about 500,000 individuals are infected each year, with approximately 50,000 deaths (Barrett *et al.*, 2003).

The range of African trypanosomiasis is restricted by the distribution of its tsetse fly vector (*Glossina spp*) and of its animal reservoirs. The tsetse fly vector infests 11 million km^2 south of the Sahara, transmitting trypanosomes to livestock, causing the disease "Nagana"; and to humans, causing sleeping sickness. Nagana is a result of infection from the species *T. brucei*, *T. congolense* and *T. vivax*. Wildlife including warthog, buffalo and bush pig are the natural hosts of these parasites. Most wildlife species and some indigenous livestock breeds can tolerate infection without major adverse consequences and some even recover spontaneously without any treatment. Fly infection rates vary but are generally around 10-15% for *T. congolense* and *T. vivax* but much lower for *T. brucei*. Once a tsetse becomes infected, it remains infective for the rest of its life. Tsetse flies inhabit a broad range of habitats from the semi-arid margins of the Sahel, through tropical rainforests to the sub-tropical savannas of S.Africa. Twenty two species and various sub-species of tsetse are recognised and these are divided into three groups: (1) Forest tsetse- *fusca* group, (2) Riverine tsetse- *palpalis* group and (3) Savanna tsetse- *morsitans* group (Boum *et al.*, 2001). Animal trypanosomiasis has a detrimental effect on the African economy and, as a consequence, indirectly on the population. Animals kept in areas of moderate risk have lower calving rates, lower milk yields and higher rates of calf mortality. In tsetse-infested areas trypanosomiasis can reduce the offtake of meat and milk by at least 50% (Swallow, 2000).

Human sleeping sickness is a result of infection by subspecies of *T. brucei* termed *T. b. rhodesiense* and *T. b. gambiense*. The former causes an acute illness in East Africa and the latter causes a chronic illness predominantly found in West and Central Africa. *T. b. brucei* is identical morphologically and biologically to these two subspecies in every aspect of its life cycle differing only in that infection is not transmissible to humans (Tait, 2000). Two further trypanosome species deserve mention, which are pathogenic to livestock and both are closely related to *T. brucei* but are not transmitted by tsetse flies. *T. equiperdum* is transmitted venereally between horses and *T. evansi*, which is transmitted by tabaniid flies (Tait, 2000).

The mode of transmission from the insect vector to the new host has been used to subdivide the genus into two groups: the Salivaria and Stercoraria. The difference between the two groups is defined in terms of the mode of transmission with the former occurring via the saliva of the insect and in the latter via the faeces. *T. cruzi* is a member of the stercoraria, the metacyclic stage being expelled with the faeces when the reduviid bug takes a blood meal, thereby gaining entry to the mammalian host by contamination of the bite site (Vickerman, 1985). A wider range of trypanosomes belong to the Salivaria group, *T. b. rhodesiense, T. b. gambiense, T. vivax* and *T. congolense*, where transmission occurs when the insect vector bites the host and discharges saliva containing metacyclics into the wound (Tait, 2000).

Within the tsetse fly, development of the trypanosome varies substantially between the three Salivarian species. In *T. congolense* development occurs in the midgut of the

3

tsetse, and then in to the mouths parts. In *T. brucei*, development in the midgut is followed by development in the salivary glands, while development of *T. vivax* occurs solely in the fly's proboscis (Vickerman *et al.*, 1991). In this study the emphasis will be on *T. brucei*.

1.2 GEOGRAPHICAL DISTRIBUTION

The distribution of sleeping sickness occurs in endemic foci. The parasites also infect wild and domestic animals that serve as reservoir hosts. For example, *T. b. gambiense* is common in pigs and *T. b. rhodesiense* is found in cattle and game. The current epidemics of the disease are caused by *T. b. gambiense*, in countries ranging from southern Sudan, northern Uganda, the Democratic Republic of Congo, Congo, Central African Republic and Angola (Fig 1.1). The severity of many of these epidemics has been heightened by the fact that due to political instability there has been a breakdown in primary health care. Trypanosomiasis is endemic in 36 of the 52 African countries. Approximately half a million people carry the disease today and in some villages 50% of the inhabitants are infected. The WHO has reported that deaths due to sleeping sickness in two provinces of the Democratic Republic of Congo at least equal those of AIDS (Masiga and Barrett, 2000). The foci of *T. b. rhodesiense* infections are in eastern and southern Africa with an epidemic in Uganda and drug resistance to Melarsoprol, the only drug available for treatment of late stage *T. b. rhodesiense* infection reported in Kenya and Uganda (Matovu *et al.*, 2001).
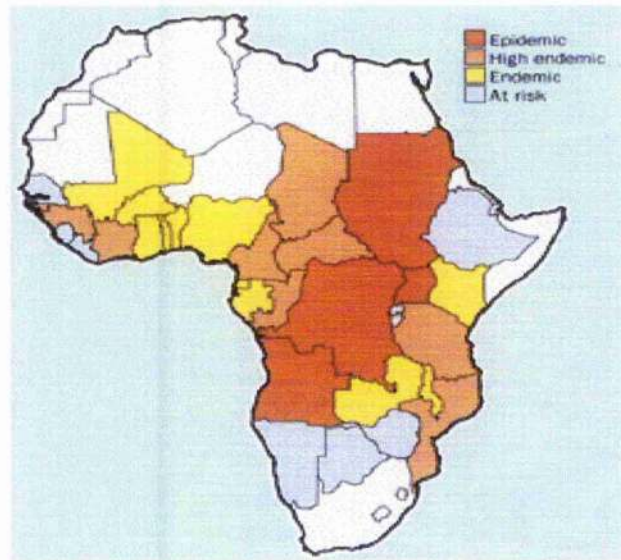
Fig 1.1: Map showing countries of Africa where trypanosomiasis is prevalent. Figure reproduced from http://www.gla.ac.uk/ibls/ii/mbp/resint.htm. (Dr M.B.P Barrett).

1.3 LIFE CYCLE

The life cycle of *T.brucei* has been thoroughly described (Vickerman, 1985). The parasite undergoes many changes as it is transmitted between the blood of a mammalian host and the gut of the tsetse fly, these being required for adaptation to the very different environmental conditions encountered (Hendrick *et al.*, 2000). In particular, the parasite undergoes metabolic development in order to successfully move from the glucose-rich bloodstream to the tsetse midgut, where proline is the main energy source (Priest and Hajduk, 1994). The trypanosome also changes the major proteins that comprise its surface coat and undergoes morphological development from the bloodstream trypomastigote to the tsetse procyclic stage (Brown *et al.*, 1973). The successive stages in the life cycle of *T.brucei* reveal that, underlying the obvious morphological alterations more complex changes in the parasite's structure are taking place. The major changes take place in the mitochondrial system and at the surface

membrane. These changes are both linked to the survival mechanisms, the former is related to the to adaptive changes in energy metabolism, the latter needed for evasion of the mammalian host's non-specific and specific defence mechanisms. It is also important to note that during the developmental cycle the trypanosome switches between proliferative phases in which it undergoes binary fission, which is linked with establishing the parasite in a new environment, and non proliferative phases in which it is incapable of division, and connected with major changes in the environment when it moves between the insect vector and the mammalian host (Vickerman, 1985).

T. brucei has six distinct stages in its life cycle. Slender form bloodstream parasites multiply by binary fission thus increasing numbers in the blood. Slender forms also transform, firstly into intermediate bloodstream forms and then to stumpy forms that are infective to a tsetse fly. In the slender forms, the mitochondrion is inactive but begins to become active in the stumpy forms. In the midgut of the tsetse fly, the trypanosomes undergo a further transformation to procyclic forms. The procyclic forms differentiate into non-dividing mesocyclic forms and migrate to the salivary glands. These then differentiate into epimastigotes, which then undergo extensive division. The cycle is complete when the epimastigotes, which are unable to survive in the mammalian host, differentiate into the non-dividing metacyclic forms (Figure 1.2). These metacyclics are found free in the saliva of the vector and are able to withstand the host's defences having acquired the variable surface glycoprotein (VSG) on their surface (Vickerman, 1985; Matthews, 1999).

The VSG coat is present only in the mammalian stages of the parasite and is lost once cyclical development in the tsetse fly begins, until the metacyclic stage in the tsetse fly is reached when recoating occurs. As well as providing a barrier to natural host defences, the VSG coat also enables the parasite to evade the host's immune response, by changing its surface coat and therefore the host cannot mount an adequate antibody response quickly enough the kill the parasites. Antigenic variation involves the replacement of one VSG coat by another, however what initiates this switch is uncertain (Vickerman, 1985).

The parasites multiply by binary fission as long slender trypomastigotes with a doubling time of approximately 6 hr. The parasitaemia fluctuates owing to evasion of the host's immune response by antigenic variation on the part of the parasite. The slender forms, referred to as "homotype" as they primarily express a single homogeneous surface antigen, form the major part of the population. When the host mounts an IgM immune response to the surface antigen homotype, the levels of parasitaemia go into remission due to the destruction of trypanosomes by the action of antibody and complement (corresponding to the variable antigen type (VAT)). In addition to the homotype (VAT) a trypanosome population also contains minor VATs or "heterotypes" which continue to multiply during the remission phase. One of these VATs overgrows the others to give rise to a recrudesence of the parasitaemia in which it becomes the homotype. This in turn leads to an increase in parasitaemia, induction of antibodies to the VAT and then remission. It is this process which gives rise to the characteristic fluctuations in

parasitaemia observed in human sleeping sickness. Each VAT is determined by a specific VSG (Vickerman, 1985).
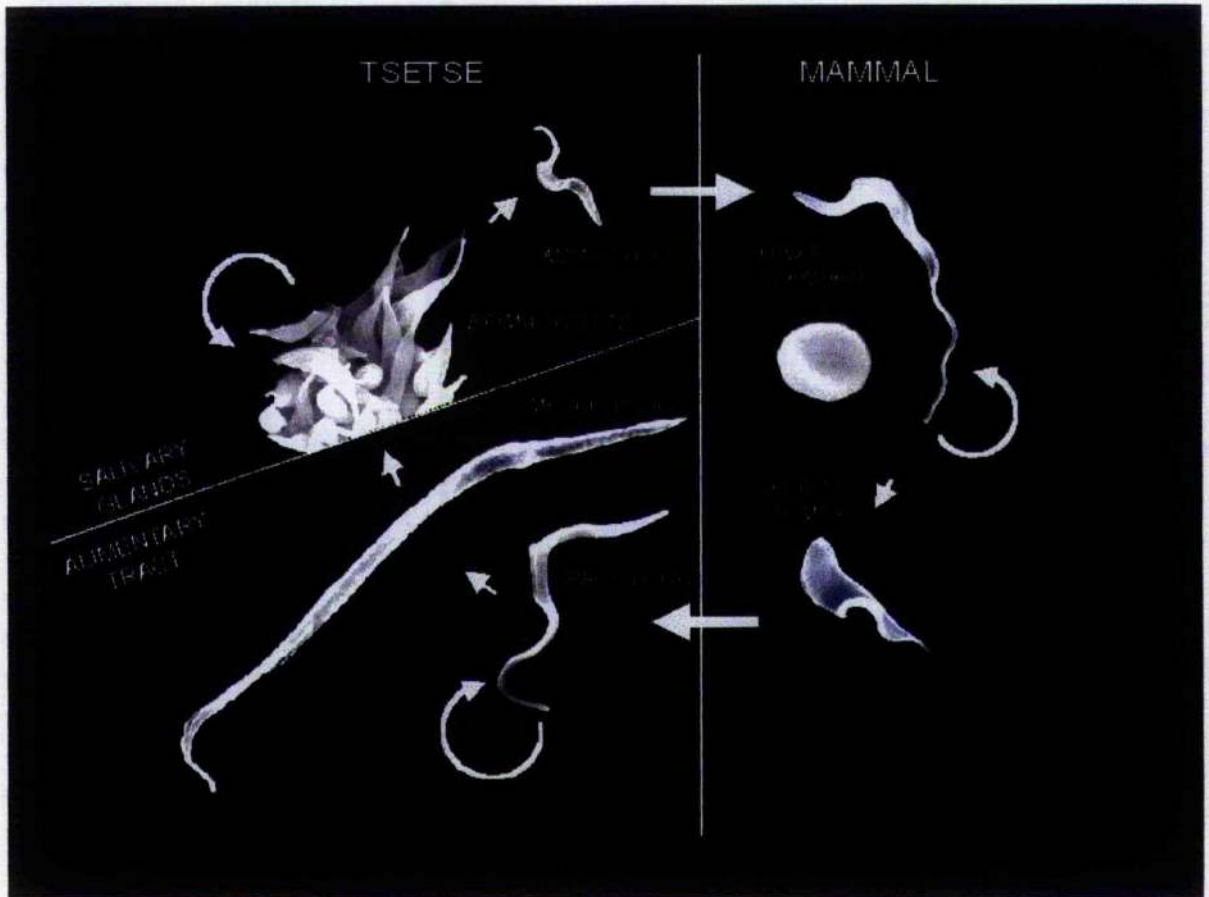
Fig 1.2: The life cycle of *T. brucei*.
Figure reproduced from http://www.gla.ac.uk/centres/wcmp/research/barry/research.html#1.
(Professor D. Barry and Dr R. McCulloch)

1.4 PATHOLOGY OF SLEEPING SICKNESS

Human sleeping sickness is an extremely debilitating disease, which is categorised into two distinct phases: early and late. On biting the mammalian host, the tsetse fly deposits metacyclic trypanosomes in the dermal connective tissue leading to a localised inflammatory response, which culminates in the development of a "chancre". From the

chancre the parasites spread to the draining lymph nodes and bloodstream, initiating the haemolymphatic early phase of the disease, characterised by general malaise, headache, fever, peripheral odema and anaemia (Masiga and Barrett, 2000). Symptoms of this early stage are relatively non-specific and can be easily confused with those of other diseases such as influenza and malaria. The important thing is the blood-brain barrier. The parasites cross it at multiple sites, including the choroid plexus and supra-chiasmatic nucleus into the brain and the cerebrospinal fluid (CSF) resulting in the late stage of the infection (Vickerman, 1985). This usually occurs within the first month post infection by *T. b. rhodesiense*, but over a period from several months to years after infection with *T. b. gambiense* (Masiga and Barrett, 2000). Subsequent neural damage and host reaction cause the more specific symptoms associated with sleeping sickness, namely, severe headaches, unusual sleep patterns, loss of concentration and coordination, leading to brain function deterioration which, if left untreated inevitably leads to coma and death (Bentivaglio *et al*, 1994).

1.5 CHEMOTHERAPY

The development of antimicrobial arsenical drugs stems from the first studies on chemotherapy conducted by Paul Ehrlich and his colleagues in 1909. In most clinical situations, the use of such compounds has been superseded by the development of more selective agents (Fairlamb *et al.*, 1989). Only a limited number of drugs are currently available for treatment of African trypanosomiasis. Ehrlichs early work established the vital general principles of chemotherapy, which subsequently led via arsenoxide to the synthesis of arsenobenzol, the first antitrypanosomal drug, followed

by the current less toxic counterparts. The discovery of suramin in the 1920s and pentamidine in the 1930s were mainly as a result of Ehrlich's earlier work on trypanosomal compounds (Gutteridge, 1985). For nearly 60 years there were no further advances in the development of novel compounds for treatment, until the 1980's, when it was discovered that eflornithine (DFMO) had trypanocidal properties (Wang, 1997).

Antimicrobial drugs are used in three distinct ways: prophylaxis, treatment and prevention of transmission from the blood. Currently chemotherapy of African trypanosomiasis centres around four main drugs: pentamidine for early stage treatment, suramin for the treatment of the early stages of infection, while DFMO and melarsoprol are effective for the treatment of late stage infection when the trypanosome have entered the CNS (Gutteridge, 1985).

The drugs listed above are plagued by numerous problems, ranging from oral inabsorption, acute toxicity, short duration of action and unfortunately the emergence of drug resistance. The ideal drug for the treatment of sleeping sickness would have the following properties: it would have high oral availability, be administered in a single dose or a limited number of doses over a short period of time, have minimal side effects but yet be active against all stages of the course of infection and all strains and species of trypanosome which are responsible for disease. The lack of oral activity and severe side effects cause enormous logistical problems. The former is most critical for a chemoprophylactic due to fact that mass administration is much more difficult if an injectable formulation has to be used. The successful treatment of African

trypanosomiasis often requires a stay in hospital, up to a month for people with late stage disease, as a consequence of the potentially dangerous side effects of treatment. However the most important limitation of current chemotherapy is the lack of drugs with a broad spectrum of activity against all stages of the disease and species of parasite. In particular, there is a lack of drugs available with activity against stages involving the CNS, and activity against *T. b. rhodesiense* is as a rule lower than that seen in *T. b. gambiense* (Gutteridge, 1985). Mechanisms of antitrypanosomal action of these drugs are largely unknown, except DFMO, which is a suicide inhibitor of ornithine decarboxylase (Carter and Fairlamb, 1993).

1.6 DRUG DESCRIPION AND MODE OF ACTION

1.6.1 Melaminophenyl arsenicals

Melarsoprol (MelB) contains a trivalent arsenic element with a highly reactive arsenoxide group (Fig 1.3). It is the presence of the arsenoxide group that allows passage of MelB across the blood brain barrier (BBB) as it confers the physical property of lipid solubility. The transport of MelB into the trypanosome is via a purine transporter (Carter and Fairlamb, 1993). This transporter, P2, whose role is the uptake of the 6–aminopurines adenine and adenosine, recognises a motif that is present on these substrates and also the melamine moiety of melarsoprol (Fig 1.3) (Carter *et al.*, 1999). Trypanosomes have a highly developed transport system due to the fact that they are unable to synthesise purines and so must acquire them from their host. MelB acts, therefore, as a competing ligand for the purine site on the transport protein (Carter and Fairlamb, 1993). This could also explain it's selective mode of action against the

trypanosome as a result of P2 transport leading to accumulation of high levels of drug within the trypanosome.

Modification of the MelB melamine ring has generated other analogous compounds. A number of trivalent arsenicals have been developed as a result of the modification of MelB namely, Melarsen oxide (MelOx), Phenylarsine oxide (PhenOx), Cymelarsan (MelCy) and Trimelarsan (MelW). MelB is a prodrug and the active metabolite in the body is MelOx (Fig 1.3). It is thought that enzymatic processes *in vivo* contribute to the conversion of MelB to MelOx (Nok, 2003). It is thought that arsenicals inhibit a range of enzyme activities *in vitro* but it is unknown what the primary target is.

MelCy (Melarsamine hydrochloride) and MelW are both trivalent derivatives of MelB both having the melamine ring (Fig 1.3). Cymelarsan has been developed with activity against trypanosomes of the *T. brucei* sub-group and has been licenced for use in camels and has proved effective against *T. evansi* both in animal infections and *in vitro* (Ross and Barns, 1996).

1.6.2 Pentamidine

Pentamidine has been the first line drug for the treatment of early stage *T. b. gambiense* infection (de Koning, 2001A). This drug is an aromatic diamidine and a di − cationic molecule and as a result diffuses slowly across biological membranes (de Koning, 2001B). Its mode of action remains a matter of debate. However, it is generally accepted that this diamidine has a limited activity towards host cells (Denise and

Barrett, 2001). The uptake of the drug seems to involve the P2 transporter, which, has also been associated with the uptake of the melaminophenyl arsenicals (Carter and Fairlamb, 1993). A biochemical reason for the transport of diverse molecules such as Pentamidine, MelB and adenosine by the same transporter is suggested by the identification of a substrate recognition motif for P2 for both classes of drugs (Fig 1.3) (Barrett and Fairlamb, 1999). Two further transporters have been shown to be involved in Pentamidine uptake, LAPT1 (low affinity pentamidine transporter) and HAPT1 (high affinity pentamidine transporter) (de Koning, 2001A).

1.6.3 Suramin

This sulphated naphthylamine was first introduced in 1922. It is generally considered the drug of choice for the treatment of early stage trypanosomiasis, especially *T. b. rhodesiense*. Due to its chemical structure and high negative charge suramin is not effective against the late stage of infection, as it cannot traverse the blood brain barrier (Wang, 1995).

Suramin has been found to bind to albumin and low density lipoproteins (LDL) in serum. Therefore, it is likely that suramin binds to a variety of serum proteins that enter the trypanosomes initially by receptor mediated endocytosis of some of the serum proteins (Wang, 1995). Suramin is a member of the sulfonated aromatic compounds found to bind to the active sites of dehydrogenases and kinases (Hawking, 1978). It has been found that in *T. brucei* suramin is a potent inhibitor of all the glycolytic enzymes in *T. brucei* (Nok, 2003).

13

It is possible that suramin may bind to the glycolytic enzymes and interfere with the import of these enzymes into the glycosomes (Clayton, 1987). Thus, inhibition of glycosomal protein import may lead to a gradual decrease of enzyme concentration in the glycosome and as a result a slowing down of energy metabolism in suramin treated trypanosomes (Wang, 1995).

1.6.4 Eflornithine

The clinical name for DL-α-difluoromethylornithine (DFMO) is eflornithine, a fluorinated derivative of ornithine that was first synthesised as a potential suicide inhibitor of ornithine decarboxylase (ODC). ODC is the key enzyme in the pathway leading to the biosynthesis of polyamines: putrescine, spermidine and spermine and these are essential for proliferation of prokaryotic and eukaryotic cells. DFMO has good antitrypanosomal activity for the treatment of both early and late stages of *T. b. gambiense* infections but unfortunately it is relatively ineffective against *T. b. rhodesiense* infections (Wang, 1995).

DFMO acts by inhibiting trypanosomal ODC. Due to the low rate of *in vivo* turnover of ODC in the trypanosomes, DFMO can effectively inhibit the enzyme activity and deplete the intracellular polyamines to bring the cells into a dormant G0 phase, which is destroyed by the host immune response. The trypanosomes are capable of taking up polyamines, but the polyamine level in mammalian plasma is extremely low due to the presence of very high levels of polyamine oxidase. This low polyamine level in the environment helps explain why, among the many species of parasitic protozoa, the

14

African trypanosomes are the only ones susceptible to DFMO treatment. This is due to the fact that they stay in the bloodstream of mammalian hosts where the level of polyamines is low (Wang, 1995).

1.7 VETERINARY TRYPANOCIDES

1.7.1 Berenil

Berenil, like Pentamidine is an aromatic diamidine and a di – cationic molecule and as a result diffuses slowly across biological membranes (de Koning, 2001B). Berenil is widely used for the treatment of *T. brucei* infections in cattle. It is transported in *T. b. brucei* by P2, but not by HAPT1 or LAPT1. It is also an effective drug against *T. congolense* infection even though this trypanosome lacks detectable P2 transporter activity (de Koning, 2001B).

1.7.2 Isometamidium

Isometamidium cloride (Samorin) is used both prophylactically and therapeutically for the treatment of animal trypanosomiasis. It is a conjugate of homidium and part of the Berenil molecule. It is thought that the action of isometamidium in *T. congolense* is dependent on two sets of transporters: facilitated diffusion in the plasma membrane and active transporters in the mitochondrial membrane. In *T. b. brucei*, the P2 transporter may be responsible, in part, for drug uptake as it inhibits P2–mediated adenosine uptake. However, adenosine does not significantly inhibit isometamidium uptake suggesting its role is not an important one (de Koning, 2001B).

1.7.3 Homidium

Homidium otherwise known as ethidium, was first found to have trypanocidal activity over 50 years ago. It is a potent mutagenic agent with many non–discriminating biochemical reactivities. Its mechanism as a trypanocidal agent is poorly understood. It has been shown to interfere with the glycosomal functions, trypanothione metabolism and replication of kinetoplast minicircles in the trypanosomes. Homidium was extensively used in the 1960s and 1970s, but its usefulness has been greatly reduced owing to widespread trypanosomal resistance. The mechanism of this resistance is unknown (Wang, 1995).

Fig 1.3: Structures of the the P2 substrate adenosine, melaminophenyl arsenicals, diamidines, phenylarsine oxide, eflornithine and suramin.
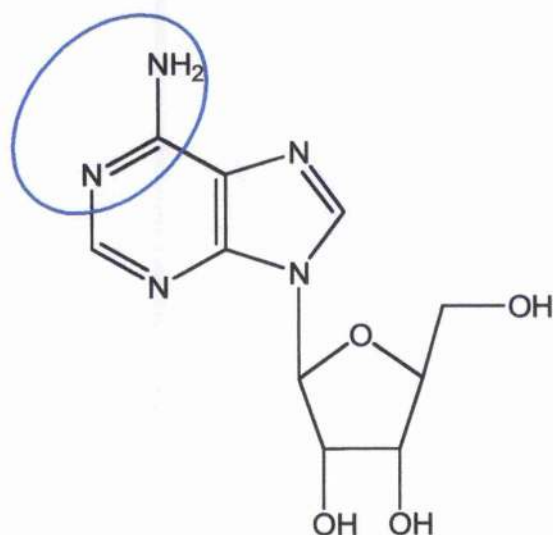
Adenosine
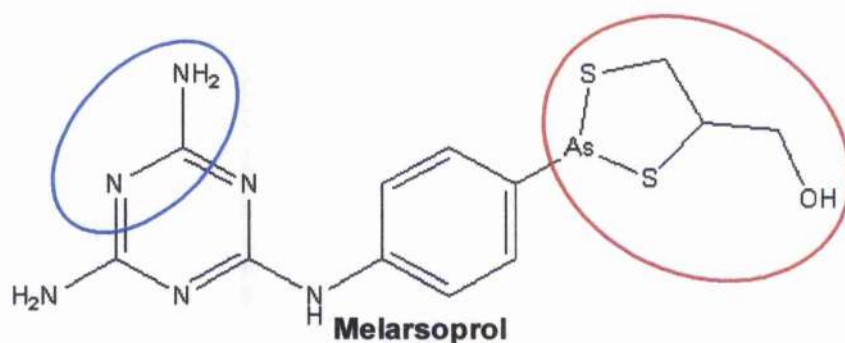
Melarsoprol
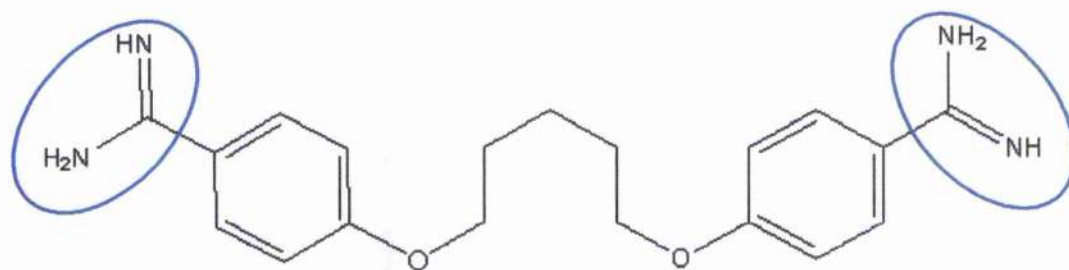
Pentamidine

Fig 1.3 cont
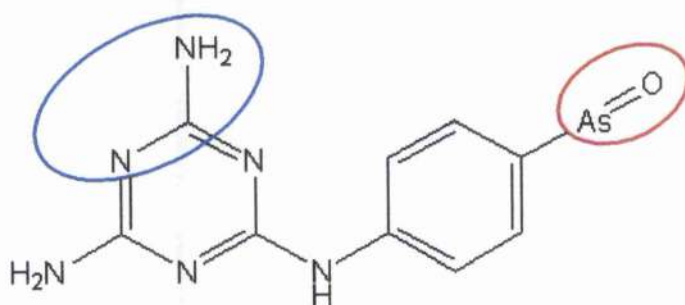
Melarsen oxide
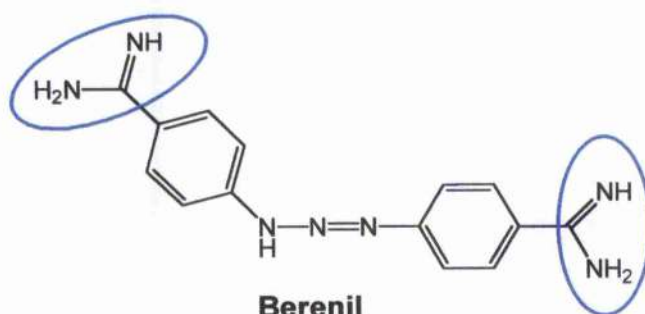
Berenil
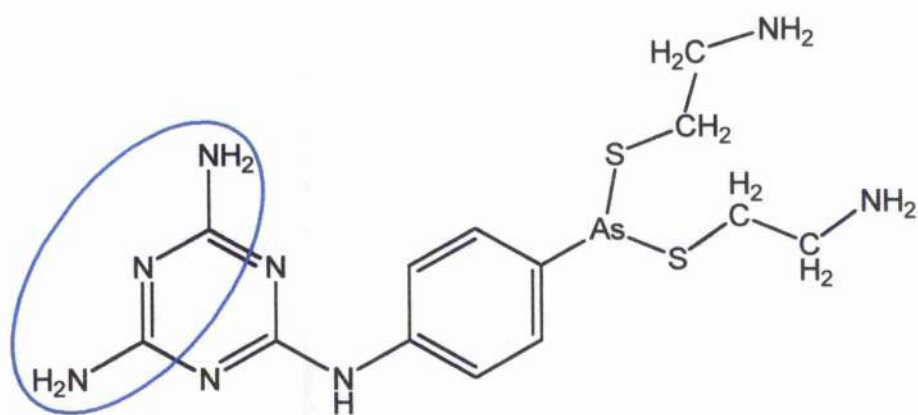
Cymelarsan

Fig 1.3 cont
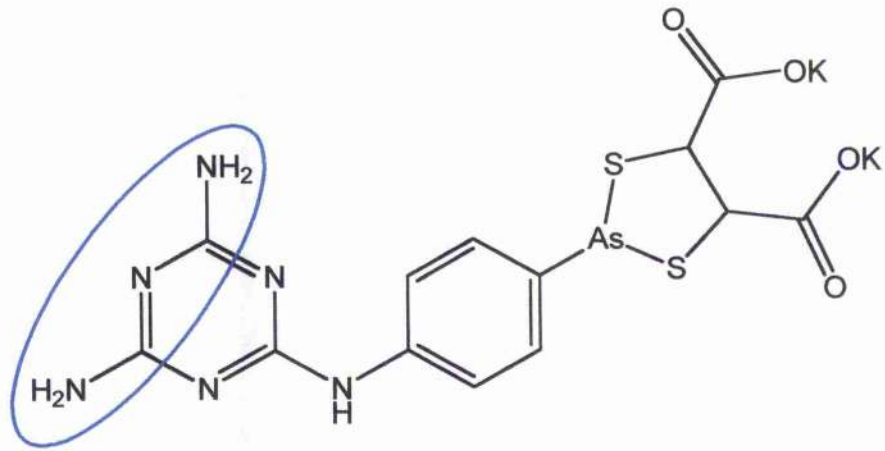
Trimelarsan
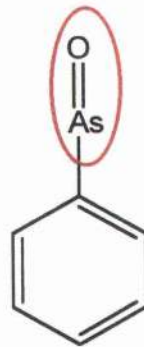
Phenylarsine oxide
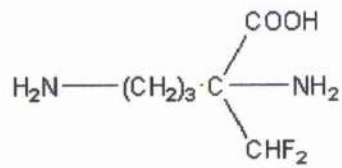
DFMO
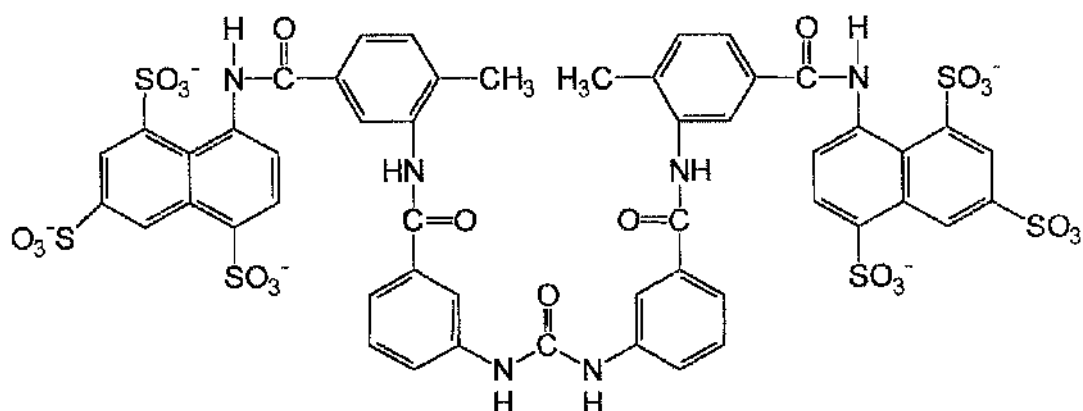
Suramin

Fig 1.3 The P2 transporters whose normal physiological role is the uptake of 6-aminopurines adenine and adenosine recognises a motif which is present on these substrates and the melamine moiety of MelB, MelCy, MelOx, MelW, Berenil and Pentamidine (de Koning and Jarvis, 1999). This recognition motif is circled in blue. The arsenoxide group that confers the physiochemical ability of lipid solubility thus allowing passage across the blood brain barrier (Pepin and Milford, 1994) is present on MelB, PhenOx and MelOx and is circled in red. Models of drug structures were kind gifts from Harry de Koning.

1.8 DRUG RESISTANCE IN *T. BRUCEI*

Drug resistance is only one of the many possibilities that may be linked to drug treatment failure with trypanocides in the field. Even if drugs are accurately administered, it would have to be demonstrated that the parasites have developed resistance to the drug as often other reasons for treatment failure are found, such as the accessibility of drug to trypanosomes in host tissues where the required concentration may not be attained (Burri *et al.*, 2000). The World Health Organisation laid down a definition in 1963 stating that, "The difference between drug resistance and treatment failure is that drug resistance inevitably leads to treatment failure, whereas treatment failure dose not necessarily indicate drug resistance. "

Chemotherapeutic intervention is continuously faced with increasing occurrence of drug resistance because all of the drugs currently used for treatment have been used for many decades. Consequently, resistance in all *T. brucei* sub-species from different areas of Africa has been reported (Table 1.1). There have been reports of treatment failure for patients treated with MelB, particularly in Sudan, Angola, Uganda and the Democratic Republic of Congo (Fig 1.4). It has been reported that, even after some of these patients were given subsequent treatment with the same drug no effect on the progression of the disease was observed (Matovu *et al.*, 2001).

Country	Trypanosome species	Resistance to
Nigeria	*T. brucei*	Berenil
Kenya	*T. b. rhodesiense*	Melarsoprol
Uganda	*T. b. rhodesiense*	Melarsoprol and DFMO
	T. brucei	Berenil
D. R. Congo	*T. b. gambiense*	Melarsoprol

Table 1.1: **Reports of drug resistance in *T. brucei*.** The reports were generated from isolated small-scale surveys and could be an under estimate of the problem (Matovu *et al.*, 2001).
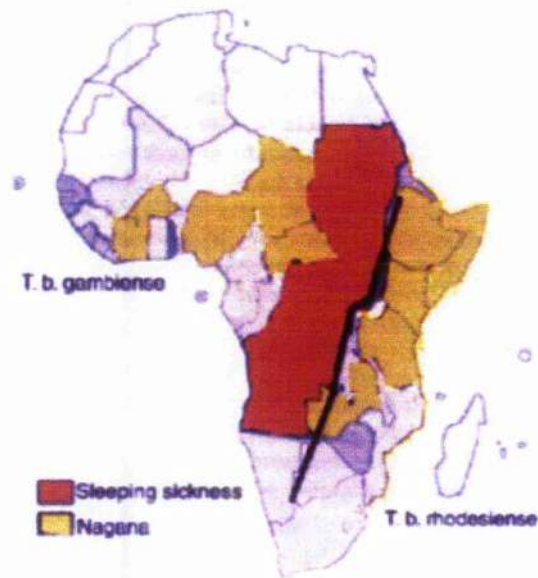
Fig 1.4: Distribution of drug resistant trypanosomes in Africa. As can be seen resistance is more widespread amongst animal trypanosome infection (Nagana), whilst human African trypanosomiasis is concentrated along the belt from Angola to the Sudan (Figure reproduced from Matovu et al., 2001).

It has been suggested that the mechanism of resistance to all melaminophenyl arsenicals is the same and occurs as a consequence of an alteration in a specific adenosine transporter (P2) that is able to recognise the melaminophenyl moiety of these trypanocides as similar to that of the purine ring of adenosine (Fig 1.3) (Carter and Fairlamb, 1993). Transport kinetic studies have shown that an arsenical-sensitive T. brucei line contains two high affinity transport systems for adenosine capable of scavenging the low concentration of nucleosides in the mammalian bloodstream (Carter and Fairlamb, 1993). The two adenosine transporters were identified: the P1 which transports adenosine, inosine and other nucleosides and the P2 which transports adenosine, adenine and melarsoprol (Fig 1.5). A melaminophenyl arsenical resistant T.brucei line (RU15) was shown to have a greatly reduced initial rate of P2 mediated

adenosine uptake. The arsenical resistance phenotype was associated with a loss of or alteration to the P2 transporter responsible for uptake of the melaminophenyl arsenical involved (Carter and Fairlamb, 1993). However, there have been several findings that suggest that alteration of the P2 transporter is not the only route by which drug resistance arises. One such investigation showed that resistance to MelCy was expressed in procyclic forms (Scott *et al.*, 1996), but it is known that the P2 transporter is not expressed in procyclic forms (de Koning and Jarvis, 1998).

Molecular and transfections studies have been carried out to determine the basis of Melarsoprol resistance in *T. brucei*. The gene encoding the P2 transporter, *T. brucei* adenosine transporter 1 (TbAT1), has been identified and expressed in *Saccharomyces cerevisiae* and was used to assess the potential of substrates to inhibit TbAT1 mediated transport (Matovu *et al.*, 2003). A gene knock–out of TbAT1 in *T. brucei* has been performed and its effect on drug uptake and resistance determined. It was found that as a result of deleting this gene, there was only a small reduction in the sensitivity of the trypanosomes to MelB, MelOx, MelCy and Pentamidine compared to the wild–type strain (Mäser *et al.*, 1999). However, resistance to Berenil increased quite significantly in comparison to the other drugs (Matovu *et al.*, 2003). The TbAT1 gene from a drug sensitive clone (STIB 777s) and a drug resistant clone (STIB 777r) was cloned and sequenced and it was found that the gene from the resistant line had 10 nucleotide differences, six of which manifested themselves at the amino acid level. These findings, therefore, provide evidence that TbAT1 encodes an adenosine transporter, which mediates the uptake of, and thus susceptibility to, melaminophenyl arsenicals and that

mutations or deletions of TbAT1 contribute to arsenical resistance in *T. brucei* (Mäser *et al.*, 1999). This result further indicates that other routes are involved in conferring the drug resistance phenotype.
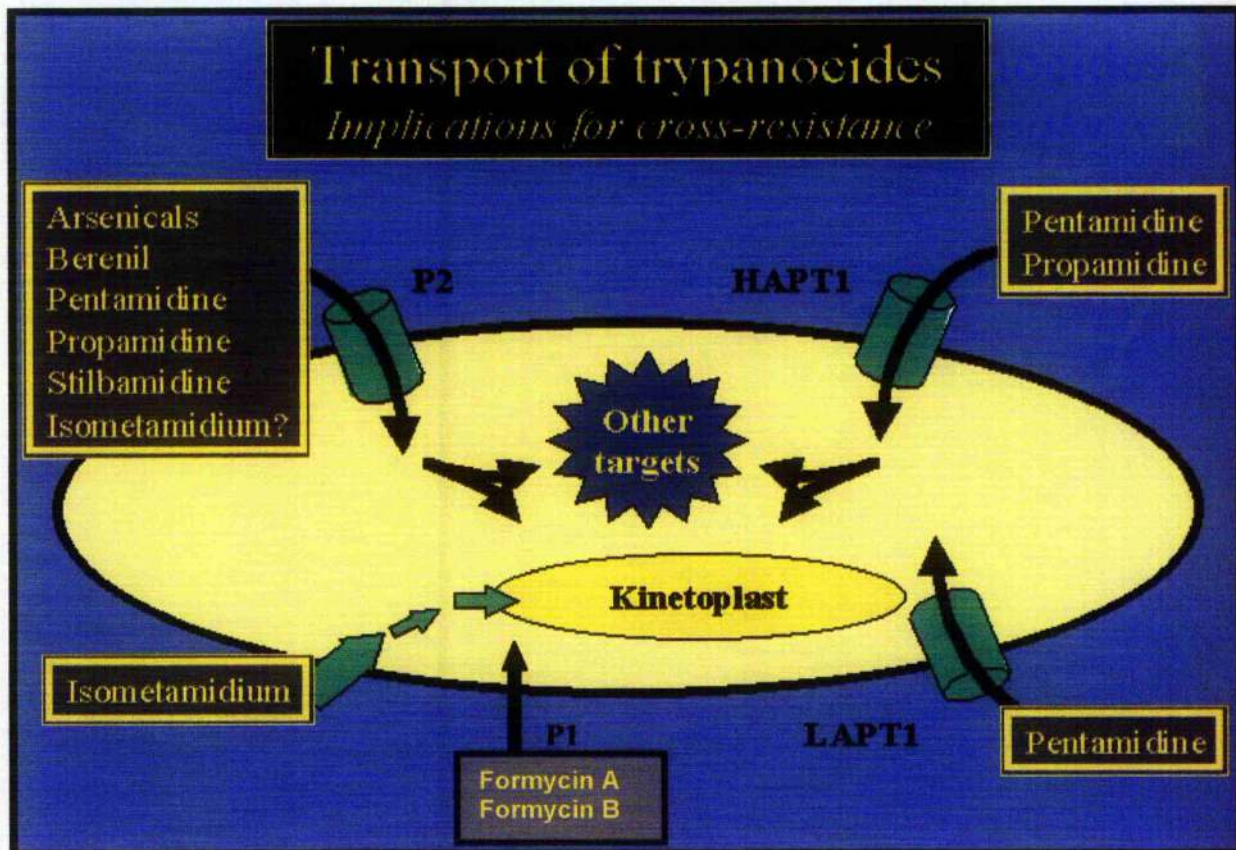
Fig 1.5: Overview of the uptake mechanisms involved in drug uptake in *T. brucei*. Indicated are the P1 and P2 transporters whose normal physiological function is the salvage of 6–aminopurines; P1 is specific for adenosine and inosine and P2, for adenosine and adenine. But the P2 transporter also mediates the uptake of arsenicals and diamidines, as shown, because of the structural similarities between the drugs and adenosine. The HAPT1 and LAPT1 are two additional transporters, which are involved in the uptake of Pentamidine (de Koning, 2001). HAPT1, high affinity pentamidine transporter; LAPT1, low affinity pentamidine transporter. Figure reproduced from: www.gla.ac.uk/ibls/ii/gifs/purmodel.gif. (Dr H. de Koning)

Most of the arsenical resistant strains analysed to date show cross resistance to all related compounds having a melaminyl moiety. However, these strains are found to be

susceptible to the lipid soluble compound phenylarsine oxide that lacks the melaminyl ring. These observations suggest that resistance is based on the melaminyl part of the molecule (Carter and Fairlamb, 1993). It has also been demonstrated that while there is cross–resistance between the melaminophenyl arsenicals and the diamidine Berenil, there is a lower level of resistance to Pentamidine (de Koning, 2001A). This has led to the proposal, first suggested 10 years ago, that Pentamidine is either taken up by a different transporter or has an altered affinity for the transporter (Fig 1.5) (Fairlamb *et al.*, 1992). Two additional transporters, specific for Pentamidine transport, have more recently been identified: the high–affinity pentamidine transporter HAPT1 and the low–affinity pentamidine transporter LAPT1; both of these transporters are adenosine insensitive (de Koning, 2001A). Although Berenil is structurally related to Pentamidine (Fig 1.3), it is not an effective substrate for HAPT1 or LAPT1, and cannot enter trypanosomes by routes other than the P2 transporter (de Koning and Jarvis, 2001). This may explain why resistance to Berenil in the field is more widespread while resistance to Pentamidine is much less common (Matovu *et al.*, 2001).

A separate study showed that a *T. equiperdum* line selected for a 36 fold resistance to Berenil also showed a four fold resistance to cymelarsan (Barrett and Fairlamb, 1999). The wild type line had an adenosine transport system similar to that of *T.brucei*. The resistant line still had both P1 and P2 activity, however the substrate affinity and uptake capacity of P2 was greatly reduced. It was concluded, therefore, that a mutation in the P2 transporter caused the parasite to become resistant to Berenil but with low cross–resistance to cymelarsan (Barrett and Fairlamb, 1999).

It has also been shown that that arsenical resistance is a stable phenotype during tsetse transmission, and this could have serious implications leading to the spread of drug resistance in the field (Scott *et al.*, 1996). In all examples of cross–resistance between arsenicals and diamidines, transport via the P2 transporter is altered in such a way that it leads to a diminished accumulation of drug and therefore confers resistance (Barrett and Fairlamb, 1999).

Resistance to Suramin does not seem to be as a result of metabolism and unlike the arsenicals and diamidines it is unlikely that the mechanism leading to resistance is a result of diminished uptake (Nok, 2003). The reason for this is that LDL uptake is essential for proliferation in *T. b. brucei*, which is unable to synthesis fatty acids and cholesterol de novo (de Koning, 2001B). A recent study has shown that suramin entry into the cell is not mediated via an LDL specific receptor and that the suramin receptor is a distinct entity. The study also showed that suramin uptake relies on prior binding to serum compounds and is by an energy–dependent mechanism that has yet to be identified (Pal *et al.*, 2002).

T. b. rhodesiense is thought to attain resistance to DFMO either by altering the properties of target enzymes or by regulating drug transport across the cell membrane (Nok, 2003). A study carried out using a DFMO resistant mutant determined that altered drug accumulation was the only mechanism that could explain the observed resistance. The drug resistant strain did not enhance its ODC activity or lower the sensitivity of its ODC toward DFMO inhibition. Also, it had the normal rate of converting exogenous

26

ornithine to endogenous polyamines. However, the mutant strain had an altered capability of taking up exogenous DFMO, accumulating it to only a very low level in the cell. This low drug concentration within the cell prevented DFMO from exerting its inhibitory effect on growth of the cells (Phillips and Wang, 1987). The possible involvement of a specific transporter protein, however has not been ruled out for DFMO uptake (Nok, 2003). Obviously more studies will need to be carried out before the mechanism of DFMO action and resistance can be fully understood.

Advances in trypanosomal chemotherapy are not very optimistic. Among the limited number of drugs currently available, each has its particular disadvantages and drawbacks and several have severe side effects. The only new drug, DFMO, displays excellent efficacy against *T. b. gambiense*, the problem is the amount of drug needed, and the requirement for hospitalisation. As yet the mechanisms of action of these drugs are generally poorly understood thus leading to a decrease in opportunities for future drug development.

On the other hand, African trypanosomes are among the most intriguing families of eukaryotic micro–organisms currently known to biologists. Many unique features are linked to their development and metabolism. These include: RNA editing, *trans*-splicing of mRNA, the kinetoplast DNA, antigenic variation, trypanothione metabolism and the glycosome. Many of these features could be potential targets for further chemotherapeutic investigations, as long as two essential requirements are met: firstly, the pathway/function is essential for the survival of trypanosome and secondly, that it is

27

unique enough that a counterpart does not exist or is sufficiently different in the mammalian host to allow for selective inhibition (Wang, 1995).

A useful strategy for understanding the mechanism of drug resistance is the genetic basis of resistance. Manipulation of gene structure and expression by gene knock-out or gene disruption followed by observations of changes in phenotype would potentially lead to a better understanding of the mechanisms of drug resistance.

1.9 TRYPANOSOMATID GENETICS

The genetics of the trypanosomal group of protozoa have been the subject of considerable research over the past 15-20 years (Smith and Parsons, 1996). A sexual cycle in *T. brucei* was discovered over 17 years ago (Jenni *et al.*, 1986). It has been demonstrated that genetic exchange occurs between trypanosomes when they infect the tsetse fly and, by marker analysis, that the process is non-obligatory to completion of the life cycle but has all the characteristics of a Mendelian system (Tait and Turner, 1990; Sternberg and Tait, 1990; Turner *et al.*, 1990; Gibson and Stevens, 1999).

A study was carried out on 17 stocks of trypanosomes isolated in Uganda. Isoenzyme analysis of these stocks indicated that the population was consistent with being diploid and in Hardy-Weinberg equilibrium. This finding provided strong evidence that these trypanosome strains were undergoing random mating (Tait, 1980). These studies preceded direct evidence of genetic exchange between trypanosome populations and assumed meiosis. Sternberg has also suggested that gene exchange may involve

meiosis and syngamy and therefore, can be explained in terms of classical Mendelian genetics (Sternberg *et al.*, 1989).

The extent to which genetic exchange occurs in natural populations, however is still a matter of debate. There are three proposed types of population structure for *T. brucei* based on the extent of sexual recombination: (1) clonal, where there is little genetic exchange, (2) epidemic, where there is some genetic exchange but it is masked by clonal expansion in some strains and (3) panmictic, where random mating occurs. The clonality theory was proposed based on isoenzyme data and evidence for strong linkage disequilibrium (Tibayrenc *et al.*, 1990). This is in contrast to the original proposal that the population structure of *T. brucei* was panmictic (Tait, 1980). These two theories are to a large extent resolved by considering an epidemic population. In this model one or two successful multilocus genotypes expand and mask the underlying occurrence of frequent genetic exchange (Maynard Smith *et al.*, 1993). A recent study using isolates from four different regions in Africa and mini and microsatellite markers has suggested that *T. brucei* populations have an epidemic genetic structure, but very well characterised human infective populations have a clonal structure (MacLeod *et al.*, 2001). Given this, it would seem that the population structure in *T. brucei* is neither clonal nor panmictic but epidemic. However, this proposal must be treated with caution because of the low numbers of isolates screened (MacLeod *et al.*, 2001).

In laboratory studies, the main limitation on the analysis of the mechanism of gene exchange in trypanosomes has been, to date, the limited number of hybrid clones

analysed. Results have indicated that the majority of hybrid clones possess the same DNA contents as their parents, supporting a conventional Mendelian system of genetic exchange. This is not the case, however, with all products of mating. One study showed that approximately 66% of hybrid progeny had 1.5 times DNA content of the parental trypanosomes. The interpretation of the results after analysis of these clones was that the DNA content reflected triploidy and that the clones arose as a result of fusion between haploid and diploid parental nuclei, which is an aberrant event in a genetic exchange mechanism involving meiosis (Gibson et al., 1992). In a separate study, analysis of 30 recombinant clones were tested for their DNA content and only two of the clones were found to have raised DNA content, 1.5 times that of the parents. Marker analysis was carried out on these two clones and one was found to be triploid due to the fact that for seven of the independent loci the clone was trisomic. The second clone had inherited a single allele from each parent for four loci and was therefore, considered diploid (Hope et al., 1999).

All laboratory investigations have been carried out using classical marker analysis of mixtures of two genetically distinct stocks in order to analyse the genotype of the progeny resulting from the co–transmission of these stocks through tsetse flies. The loci can be considered in two categories; firstly, where the parents are homozygous for different alleles then the products of mating will be either (1) heterozygous or (2) homozygous and identical to one or other parent. Secondly, where one or both parents are heterozygous for alleles at several loci, thus the products of mating will be, (1) identical to one or other parent or (2) show segregation of alleles at each locus with

heterozygous and homozygous progeny (Sternberg and Tait, 1990). The observation of products from crosses that remain identical to one or other parent indicates that genetic exchange is non–obligatory. The finding of progeny that have become heterozygous at loci where the parents were homozygous indicates that hybrid formation has occurred. The segregation of alleles at heterozygous loci indicates that mating is Mendelian.

1.10 MINI AND MICROSATELLITE MARKERS

Three minisatellite loci were used to analyse genetically distinct stocks and hybrids; MS42 localised on Chromosome I, CRAM localised on Chromosome X and 292 localised on Chromosome III. Allelic variation at each of these loci has been detected involving alleles of different size shown to represent differences in the number of repeat units (MacLeod *et al.*, 1999).

Analysis of the inheritance of this allelic variation in crosses between stocks of STIB (Swiss Tropical Institute, Basel) 386, STIB 247 and TREU (Trypanosome Research Edinburgh University) 927 has indicated that the alleles are inherited in a Mendelian manner with each F1 progeny clone inheriting a single allele from each parent (MacLeod *et al.*, 1999). Two microsatellite markers, PLC and JS2 (MacLeod *et al.*, 2000), which localised on chromosomes II and V respectively also showed allelic variation and segregation at these loci. The implication from this finding is that inheritance of each individual alleles can be traced in the progeny clones between crosses by exploiting the different sized alleles for all three minisatellites, MS42, CRAM and 292 and the two microsatellite markers PLC and JS2.

There have been 90 candidate polymorphic microsatellites identified for chromosome I as a result of sequencing the *T. brucei* stock Tb927 by the Sanger institute. With these microsatellites a genetic map of chromosome I has been constructed. Crossover frequencies for chromosome I range from ~8 to >90 kb/cM, which gives an average physical size for the recombination unit of 22 kb/cM. This is similar to that found for *P. falciparum* (Hall *et al.*, 2003). A genetic map has also been constructed for chromosome II, where the physical size for the recombination unit was 12.1 kb/cM (El-Sayed *et al.*, 2003). These maps are essential for determining the inheritance of each allele in the progeny clones and eventually the localisation of a trait of interest to a defined region on a chromosome.

1.11 GENETIC ANALYSIS OF PHENOTYPE

Genetic analysis of phenotypic traits is a tool that can be used to define the function of genes identified by sequencing projects of various parasitic protozoa. Genetic techniques can be divided into two classes, forward and reverse, which is dependent on whether analysis is started at the level of phenotype or gene, respectively. There are two approaches that can be used for forward genetic analysis of phenotype, firstly classical genetic analysis where the genetic basis of existing variation in phenotype can be determined and secondly the generation of mutants and subsequent genetic analysis to identify the genes.

The classical genetic approach has been used in this study to determine the genetic basis of drug resistance. This approach uses naturally occurring variation (drug

resistance/sensitivity) between trypanosome stocks followed by crosses to determine the genetic basis of variation using segregational analyses in the F1 progeny. The three trypanosome stocks were initially isolated from the field and cloned lines of these stocks were shown to differ in their sensitivity to killing by Cymelarsan where STIB 386 and TREU 927 were resistant in comparison to STIB 247 *in vivo* (Table 1.2) (Scott *et al.*, 1996; Tait *et al.*, 2002). Using the results obtained from the segregation analyses a genetic model of inheritance of phenotype can be proposed and tested by either a backcross or by the generation of F2 progeny.

Details of Parental stocks					
Stock Number	Place of Isolation	Host	Species	Cymelarsan Resistance	Year of Isolation
STIB 247	Tanzania	Hartebeest	*T. b. brucei*	S	1971
STIB 386	Ivory Coast	Man	*T. b. gambiense*	R	1978
TREU 927	Kenya	Tsetse fly	*T. b. brucei*	R	1969

Table 1.2: Details of each parental trypanosome stock used in this investigation as described in Turner *et al.*, (1990). The Cymelarsan resistance data was based on an in vivo assay (Scott *et al.*, 1996). S, sensitive; R, resistant.

Genetic mapping can be used to define loci that contribute to heritable phenotypes in organisms that undergo sexual recombination. This approach uses genetic maps comprising of polymorphic markers that can be scored easily at numerous loci to demarcate a unique pattern of heritable alleles, known as haplotypes, for each individual progeny clone. The co-segregation of specific marker alleles and a phenotype can then allow a chromosomal region to be linked to the trait of interest. In contrast to biochemical approaches examining protein pathways to gene discovery, genetic

mapping uses the association between genotype and phenotype to determine markers linked to a particular trait. As a result, this requires no prior knowledge of the biochemical processes that give rise to the trait although it can be used to test a hypothesis about the role of a specific gene in conferring a phenotype (Ferdig and Su, 2000).

In order to identify the genes involved in determining the trait of interest, linkage analysis is required using molecular markers in established genetic maps and consequently the region of the genome where the gene lies can be identified. As the genome sequence of *T. brucei* is nearing completion (http//:www.tigr.org: http//:sanger.ac.uk) with chromosomes I and II annotated (Hall *et al.*, 2003; El-Sayed *et al.*, 2003, respectively) and a nearly complete genetic map has been made (MacLeod and Tweedie, unpublished results) then with polymorphic markers at defined locations on the physical map, it is possible to map a locus determining a particular phenotype to a particular physical region on the genome. Based on the flanking linked markers, it is then possible to define the candidate open reading frames that define the locus. If the region between the flanking markers is relatively large, >100 kb then fine mapping within this defined region can be undertaken in order to narrow the region further and so reduce the number of candidate genes involved in determining the trait of interest. At this point a reverse genetic approach can be undertaken, which involves knocking out each candidate gene or allele in turn and determining if there is a change in phenotype in the parental stock.

There have been a number of genetic mapping studies conducted to identify the determinants causing particular traits in parasitic protozoa. Perhaps the most widely studied and extensive of these is chloroquine resistance in *P. falciparum*, where it is thought that resistance is determined by mutations in the *pfcrt* gene. A cross between a chloroquine resistant clone (Dd2) and a chloroquine sensitive clone (HB3) was undertaken and linkage mapping localised the chloroquine resistance determinant to a 36 kb segment of *P. falciparum* chromosome 7. Further analysis ruled out the role of the *pfmdr* 1 gene as a determinant of chloroquine drug resistance (Su *et al.*, 1997). Instead, evidence was obtained that mutations in a highly interrupted and previously undetected gene, *pfcrt*, in the 36 kb segment was involved in conferring chloroquine resistance (Fidock *et al.*, 2000). Translation of the Dd2 *pfcrt* coding region produced a 424 amino acid, 48.6 kDa protein and database searches revealed that PfCRT belonged to a family of putative transporters or channels with ten transmembrane domains (Tm).

Sequence comparisons of the *pfcrt* gene from strains Dd2, HB3 and two progeny clones revealed eight codon differences between the chloroquine resistant and sensitive clones which localized in or near the Tm domains (Fidock *et al.*, 2000). To determine whether these mutations in pfcrt were sufficient to confer chloroquine resistance, allelic exchange was undertaken to replace the *pfcrt* allele of a chloroquine sensitive line with the *pfcrt* allele from a chloroquine resistant line. Phenotypic analysis showed that the transfected mutant *pfcrt* allele conferred a chloroquine resistant phenotype to the chloroquine sensitive *P. falciparum*. All the transfected cell lines grew in 80 – 100 nM

chloroquine which is the level previously proposed as a threshold diagnostic of *in vivo* resistant lines (Sidhu *et al.*, 2002).

Genetic analysis has also been carried out in *Toxoplasma gondii* (Su *et al.*, 2002 B) and *Eimeria tenella* (Shirley and Harvey, 2000). Studies on the inheritance of virulence in *T. gondii* have been conducted using 26 recombinant progeny derived from genetic crosses between a virulent and non–virulent strain using 112 polymorphic markers. Linkage analysis and fine mapping have identified that the gene controlling virulence lies between two markers on chromosome VII (Su *et al.*, 2002). Similarly, the gene controlling resistance to arprinocid in *E. tenella* has been inked to a region on chromosome I (Shirley and Harvey, 2000). These results are discussed further in Chapter 5.

Genetic dissection of potentially complex traits, such as development, virulence, transmission and drug resistance, is now being undertaken. In the years since it was recognized that genetic inheritance could be traced with naturally occurring DNA sequence variation, the identification of genes responsible for simple Mendelian traits has become a straightforward but labour intensive task. Over 500 such genes have been mapped to specific chromosomal regions in the human and over 60 of these have been cloned based on their position in the genome (Lander and Kruglyak, 1995).

In terms of genetic mapping of a particular phenotype, two central questions need to be addressed in order to establish the feasibility of the study: 1) sufficient numbers of

progeny to enable accurate map construction and 2) the demonstration of crossing over, in conjunction with the determination of the physical size of the recombination unit. The number of progeny affects the ability to determine statistical significance of a segregating phenotype in relation to the number of loci or alleles determining the trait of interest and also, the level of resolution of a genetic map. At the beginning of this study there were 37 hybrid progeny available, which were previously generated from genetic crosses between the three parental stocks in all pair–wise combinations (Fig 1.6) (Turner *et al.*, 1990; Tait and MacLeod, unpublished results). The size of the recombination unit determines the number of markers required for a map in order to determine statistically significant linkage within a specified and workable physical distance for the trait of interest (Tait *et al.*, 2002).

TREU 927

F1 (18)   **F1 (7)**

STIB 247   **STIB 386**

F1 (12)
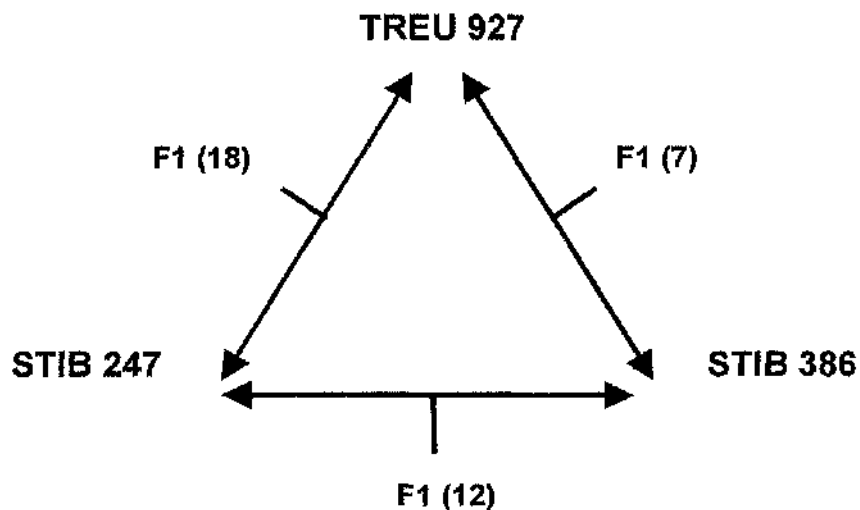
Fig 1.6: The three stocks used for the generation of a panel of F1 progeny. Crosses were performed in all pair-wise combinations of the three stocks. Numbers in brackets indicate the number of unique F1 progeny available for analysis for each cross.

1.12 OVERALL AIMS

The aim of this project was to undertake the genetic analysis of arsenical drug resistance in order to gain a better understanding of the mechanisms of resistance in *T.brucei.* Analyses was conducted to identify the genes involved in arsenical resistance using a classical genetic approach.

The project addresses the following questions:

1. Is the genetic mechanism of resistance inherited in a dominant or recessive manner?

2. What numbers of genetic loci and alleles are involved?

3. Where in the genome are the genetic loci determining drug resistance?

4. Are the resistance genes identified in the laboratory of any relevance in the field?

Before any of these questions were addressed, the number of F1 hybrid progeny derived from each cross needed to be increased as the number of progeny affects the statistical significance of segregating phenotypes and the resolution of the genetic maps. The procedure for generating F1 hybrid progeny is the subject of the following Chapter.

CHAPTER 2

GENERATION OF UNIQUE HYBRID LINES *IN VITRO* AND *IN VIVO*

2.1 INTRODUCTION

Our current understanding of the mechanism of genetic exchange in *T. brucei* is constrained by the numbers of independent hybrid progeny available from any single cross and the currently available progeny are summarized in Table 2.1. Low numbers of progeny prevent the formal demonstration that inheritance is Mendelian and the accuracy and robustness of genetic maps are reduced, which in turn limits our ability to undertake linkage analysis of a phenotypic trait of interest.

The first genetic cross demonstrated hybrid cell formation (Jenni *et al.*, 1986), but exhaustive marker analysis indicated that the three clones isolated were all vegetative products of a single mating event (Table 2.1) and were all triploid, with DNA contents greater than the parental clones. A number of subsequent crosses have confirmed hybrid formation and demonstrated allelic segregation and assortment of alleles at unlinked loci (Sternberg *et al.*, 1989 and Turner *et al.*, 1990), which is consistent with Mendelian inheritance (Tait *et al.*, 2002). A formal definition of Mendelian inheritance requires, however, agreement with theoretically predicted segregation ratios. The numbers of hybrids available are not adequate to support statistically robust agreement with predicted segregation ratios. Thus an increase in the numbers of independent progeny available from a cross would provide the statistical power to permit an analysis as to whether genetic exchange in *T. brucei* is indeed Mendelian.

	Numbers of progeny			
Cross	Total	Hybrids	Products of independent mating events	Reference
247 x 386	10	3	1	Jenni *et al.*, 1986
247 x 386	4	1	1	Sternberg *et al.*, 1988
247 x 386	>80	8	8	Sternberg *et al.*, 1989
247 x 386	11	5	4	Turner *et al.*, 1990
247 x 386	24	19	12	MacLeod and Tait (Unpublished)
247 x 927	36	24	18	Turner *et al.*, 1990
386 x 927	18	9	3	Turner *et al.*, 1990
386 x 927	62	26	4	MacLeod and Tait (Unpublished)
247 x 777		4		Schweizer *et al.*, 1994
058 x 196	10	5	3	Gibson, 1989
196 x J10	25	12	2	Gibson and Garside, 1991
058H x KP2N	91	29	2	Gibson and Bailey, 1994
Self fertilisation				
247 x 386	27	5	1	Tait *et al.*, 1996
TH2N x 058H		5		Gibson *et al.*, 1997
Backcross				
058H x P20		13		Gibson *et al.*, 1995

Table 2.1 A summary of *T. brucei* genetic crosses. As can be seen from columns 3 and 4 each hybrid clone is not necessarily a product of a unique mating event. For example in the cross conducted by Jenni (1986) three hybrid progeny were detected but as all three had exactly the same genotype for a series of markers these hybrid were the vegetative derivatives of a single mating event.

A further limitation is more directly related to the subject of this thesis, which is that the small numbers of hybrids limits the resolution of a genetic map and potential linkage

analysis of a phenotype to a locus within that map. This limitation arises because mapping is determined by the number of recombination events detected between linked loci, which is in turn a product of the average recombination ratio and the number of progeny screened.

The procedure for making a genetic cross between cloned stocks is shown in Fig 2.1 where flies harbouring mixed infections are identified by marker analysis of progeny populations. From previous studies it is known that flies with mixed infections are also likely to contain the products of crossing (Jenni *et al.*, 1986; Sternberg *et al.*, 1988; Gibson, 1989; Tait *et al.*, 1989). Individual trypanosome clones obtained from a tsetse fly carrying a mixed infection, are then isolated and expanded in mice so that they can be subjected to marker analysis and phenotype screening. The available data show that the products of mating are the equivalent of the F1 progeny of a Mendelian genetic system.
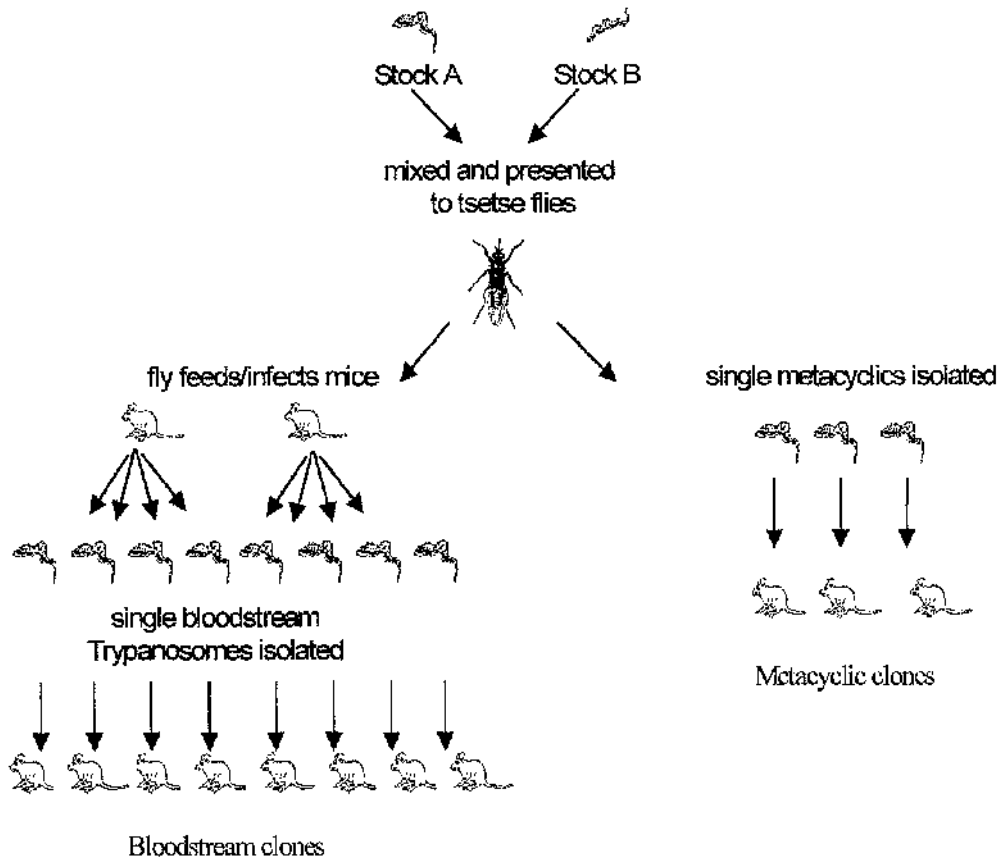
Fig 2.1 : **The procedure for generating a genetic cross in *T. brucei*.** Two genetically distinct *T. brucei* stocks are mixed and fed to tsetse flies. Flies harbouring mature infections are fed on mice, which then develop infections, the parasites in which can be screened for the presence of hybrid cells. Single trypanosomes are isolated optically either from metacyclic populations or from infected blood and each inoculated into another mouse.

Two methods have been used to isolate cloned products of crosses. Firstly, clones derived directly from the salivary glands of the tsetse fly (metacyclic clones). The resulting clones have been genotyped using a range of polymorphic markers in order to

define hybrid progeny and then determine which were new and unique genotypes. A relatively low number of F1 progeny were identified (27%) but 80% of these were genotypically unique (Tait *et al.*, 2002). Secondly, a fly carrying a mixed-infection was allowed to bite a mouse. Single trypanosomes were then isolated from the resultant infected bloodstream population and single trypanosomes from this population inoculated into separate mice followed by genotyping of each clone. Using this method 80% of the clones were F1 progeny but only 60% were unique. As the proportion of progeny was higher in the bloodstream clones, despite the fact that there were a lower number of unique genotypes, this approach generated a larger number of independent progeny and ultimately a larger set of unique clones required for linkage analysis (Tait *et al.*, 2002). Thirty-seven unique hybrid clones were previously available derived from crosses between the three parental lines (Table 2.2) (MacLeod, 1999). However these numbers needed to be increased to provide the statistical significance required for determining linkage to a trait of interest.

Table 2.2: Genotypically unique F1 hybrid progeny previously generated from genetic crosses.

Genotypically unique trypanosome F1 hybrids derived from genetic crosses							
					Markers		
Identification	Lysate No.	GUP No.	CRAM	292	MS42	JS2	PLC
Parental 247			1 - 1	5 - 5	5 - 5	5 - 6	5 - 5
Parental 386			1 - 2	1 - 2	1 - 2	1 - 2	1 - 2
Parental 927			3 - 4	3 - 4	3 - 4	3 - 4	3 - 4
Cross 247 x 927							
F124/28 bscl C5	71	4635	1 - 4	3 - 5	4 - 5	4 - 6	4 - 5
F124/28 bscl B3	73	4923	1 - 4	3 - 5	4 - 5	4 - 6	3 - 5
F974/70 mcl 4	77	3086	1 - 4	3 - 5	4 - 5	3 - 5	4 - 5
F532/63 bscl 2	80	4630	1 - 4	3 - 5	3 - 5	3 - 6	4 - 5
F532/63 bscl 3	78	4925	1 - 4	4 - 5	4 - 5	4 - 5	4 - 5
F532/63 bscl 7	79b	4631	1 - 4	4 - 5	4 - 5	3 - 6	4 - 5
F532/63 bscl 5	81	4634	1 - 3	3 - 5	4 - 5	3 - 6	4 - 5
F532/72 mcl 5	88	4392	1 - 4	4 - 5	4 - 5	3 - 5	3 - 5
F532/72 mcl 1	84	4391	1 - 4	4 - 5	3 - 5	3 - 5	3 - 5
F532/72 mcl 2	85	4636	1 - 4	4 - 5	3 - 5	4 - 5	4 - 5
F532/72 mcl 3	86	4427	1 - 4	4 - 5	4 - 5	4 - 5	3 - 5
F532/72 mcl 6	89	4393	1 - 3	3 - 5	3 - 5	4 - 5	4 - 5
F532/72 mcl 7	90	4628	1 - 4	4 - 5	4 - 5	4 - 6	3 - 5
F532/72 mcl 8	91	3538	1 - 4	3 - 5	4 - 5	4 - 5	3 - 5
F532/63 cl 16	171		1 - 4	3 - 5	4 - 5	3 - 5	3 - 5
F532/72 mcl 9	92	4366	1 - 4	4 - 5	4 - 5	3 - 6	3 - 5
F532/63 cl A11	170		1 - 3	4 - 5	3 - 5	3 - 6	4 - 5
F532/63 bscl A14/1	346		1 - 3	3 - 5	4 - 5	3 - 6	3 - 5
Cross 247 x 386							
F9/45 mcl 2	30	3300	1 - 2	1 - 5	2 - 5	1 - 5	1 - 5
F9/45 mcl 10	34	5060	1 - 1	2 - 5	1 - 5	1 - 6	2 - 5
F9/45 mcl 11	35	3287	1 - 2	1 - 5	1 - 5	1 - 5	2 - 5
B8O cl 2	162		1 - 2	2 - 5	2 - 5	2 - 6	2 - 5
F492/50 bscl 1	174	174	1 - 1	2 - 5	1 - 5	2 - 6	2 - 5
F492/50 bscl 5/1b	332	5316	1 - 2	1 - 5	1 - 5	2 - 6	2 - 5
F492/50 bscl 8	181	5317	1 - 1	2 - 5	1 - 5	1 - 6	2 - 5
F492/50 bscl 9	182	4688	1 - 2	1 - 5	2 - 5	2 - 5	2 - 5
F492/50 bscl 12	185		1 - 1	2 - 5	2 - 5	2 - 6	1 - 5
F492/50 bscl 18	191	4949	1 - 2	1 - 5	1 - 5	2 - 6	2 - 5
F492/50 bscl 21	194	4950	1 - 1	1 - 5	1 - 6	2 - 6	2 - 5
F492/50 bscl 23	196	4954	1 - 2	1 - 5	1 - 5	2 - 5	2 - 5

Table 2.2 cont

Genotypically unique trypanosome F1 hybrids derived from genetic crosses							
			Markers				
Identification	Lysate No.	GUP No.	CRAM	292	MS42	JS2	PLC
Parental 247			1 - 1	5 - 5	5 - 5	5 - 6	5 - 5
Parental 386			1 - 2	1 - 2	1 - 2	1 - 2	1 - 2
Parental 927			3 - 4	3 - 4	3 - 4	3 - 4	3 - 4
Cross 386 x 927							
F296/39 22/1	264		1 - 4	1 - 3	1 - 3	1 - 4	2 - 3
F296/39 24/1	269		1 - 4	2 - 3	1 - 3	2 - 3	1 - 3
F296/39 bscl 7	207		1 - 4	2 - 3	1 - 3	2 - 3	1 - 3
F296/39 bscl 9	209		1 - 4	2 - 3	1 - 3	2 - 3	1 - 3
F296/44 bscl 1	2	3199	2 - 3	2 - 3	1 - 3	1 - 3	2 - 3
F296/44 bscl 3	6	3201	1 - 3	1 - 3	1 - 3	1 - 4	2 - 3
F296/44 bscl 4	5	3204	1 - 4	1 - 4	1 - 4	1 - 3	1 - 3

Table 2.2: All genotypically unique F1 hybrid progeny previously generated from genetic crosses (MacLeod, 1999). The cross from which each clone is derived and their individual identification numbers are listed in column 1, and these are used throughout the study. The nomenclature follows that of Sternberg *et al.*, 1989. For example F296/39 bscl 7, was the seventh clone isolated from fly 296, on day 39 post infection. Columns 2 and 3 give additional identification numbers i.e. stabilates and lysate numbers, where available. Columns 4-7 give the results of the mini and microsatellite marker analysis. Alleles are numbered 1-5 for the minisatellites, CRAM, 292, MS42 and PLC, and 1-6 for the microsatellite JS2. GUP, Glasgow University Parasitology. Bloodstream clones from the same tsetse fly, sampled on the same day and conferring the same genotypic pattern were considered to have arisen from one sexual recombination event and so were represented by one clone in the above tables.

Since uncloned bloodstream trypanosomes from crosses were already available as frozen stabilates, I used the second approach to generate new clones from pre-existing crosses, thereby increasing the number of hybrid clones to map the drug resistance trait. This chapter describes the methods used to produce new unique hybrid progeny from such crosses and define their genotype. The nomenclature for clone identification used throughout this chapter follows that of Sternberg *et al* (1989), for example F532/72 bscl 1, is the first bloodstream clone derived from fly 532, on day 72 post infection.

Marker analysis of hybrid clones has previously relied on isoenzyme, RFLP and karyotype analysis (Sternberg *et al.*, 1989; Turner *et al.*, 1990; Gibson, 1989; Gibson and Bailey, 1994). This study, however, used the highly informative polymorphic mini and microsatellite markers CRAM, MS42, 292, PLC and JS2 (Lee *et al.*, 1990; Lee *et al.*, 1994; Barrett *et al.*, 1997; MacLeod *et al.*, 1999; Sasse, 1998; MacLeod, 1999; MacLeod *et al.*, 2000) to analyse the clones derived from each genetic cross. Each of these mini and microsatellite markers are located on different chromosomes and so are unlinked and PCR based methods have been developed for detecting variation at these loci using crude lysates of procyclic or bloodstream clones as templates (MacLeod *et al.*, 2000). They are genetically easily interpreted markers, each being able to distinguish between two to four alleles in the parental clones. These markers would, therefore, allow the theoretical identification of 64 possible genotypes for the F1 progeny clones of each of the crosses STIB 247 x TREU 927 and STIB 247 x STIB 386.

2.2 MATERIALS AND METHODS

A number of solutions, buffers and media were used throughout this study, the components of each of these are listed in Appendix A1.

2.2.1 PREPARATION OF DNA FROM TRYPANOSOMES

2.2.1.1 Crude Lysates

Crude lysates were prepared from 200 µl of procyclic cultures in log phase of growth at a density of $\sim 1 \times 10^6$ trypanosomes / ml. The cells were centrifuged at 1,100 g for 7 min at 4°C, the supernatant discarded and the pellet re-suspended in 200 µl of Phosphate buffered saline (PBS) and centrifuged again at 2,500 g for 5 min. This washing process was repeated 3 times. The final pellet was then re-suspended in 50 µl lysis buffer and 0.64 mg/ml of proteinase K and incubated overnight at 56°C. The proteinase K was heat inactivated the following day at 95°C for 5 min. The lysates were then diluted 1/50 in deionised water and stored at -20°C. One µl of lysate was then used as template in subsequent PCR reactions.

2.2.1.2 DNA extraction from trypanosomes

For DNA preparations the following method was used. Lysates were prepared from 200 µl of procyclic cultures in log phase of growth at a density of $\sim 1 \times 10^6$ trypanosomes/ ml The cells were centrifuged at 1,100 g for 15 min at 5°C. The pellet was re-suspended in 450 µl of DNA extraction buffer with 50 µl of 10% SDS and 25 µl (10 mg/ml) proteinase K added. The DNA pellet was then incubated with gentle agitation at 37°C for 2hr. 500 µl of liquid phenol was added and vortexed until the solution became milky in

appearance. The mixture was separated by microcentrifugation for 5 min at 13,000 g. The top aqueous layer was then transferred to a sterile tube and an equal volume (500 μl) of 50:50 phenol/chloroform was added, vortexed thoroughly and centrifuged as before. The upper layer was transferred to a fresh tube and 40 μl of 3 M sodium acetate plus 1 ml of 100% ice-cold ethanol was added and mixed by gentle inversion, left at -20°C for 30 min and then centrifuged for 15 min at 13,000 g to pellet the DNA. The ethanol was removed, the pellet washed with 70% ethanol, left to dry in a flow cabinet, re-suspended in sterile double distilled water and then stored at -20°C.

2.2.2 Trypanosome stocks and mice

Three cloned trypanosome lines were used as parents in this study. These were STIB (Swiss Tropical Institute, Basel) 247, STIB 386 and TREU (Trypanosome Research Edinburgh University) 927. The line STIB 247 was originally isolated from a hartebeest, STIB 386 was isolated from a man and TREU 927 was isolated from a tsetse fly (Turner *et al.*, 1990).

A number of previously uncloned bloodstream populations derived from genetic crosses between the three parental lines above were also used for this investigation. Using the nomenclature described previously these uncloned stocks were, F124/28, F532/72 and F974/78 (STIB 247 x TREU 927), F9/41, F19/31, F28/46, F57/40 and F29/46 (STIB 247 x STIB 386). Routine growth and *in vivo* cloning of trypanosomes were carried out in adult female ICR mice. Immunosuppression of mice was conducted 24 hr prior to *in vivo*

cloning of trypanosomes using cyclophosphamide at a dose of 150 mg/kg by intraperitoneal (i.p.) inoculation (Scott *et al.*, 1997)

2.2.3 Optical cloning

Bloodstream form clones were produced by direct observation of single trypanosomes, under an inverted microscope, from the blood of an infected mouse. Blood was collected from the mouse by cardiac puncture into 150 μl of Carter's Balanced Salt Solution (CBSS) and heparin. A drop was added to a 1:1 solution of guinea pig serum (GPS) (Sigma) and phosphate buffered saline with 1% glucose (PBSG). Drops were applied to the centre of each well in the plate, using the tip of a paper clip, making sure the drop did not touch the side. Dilutions of the blood in GPS+PBSG were carried out until a single trypanosome was observed from a drop of the mix observed in a well of a humidified Terasaki plate.

After detection of a single trypanosome, 10 μl of fresh GPS+PBSG was added to the well and then taken up into a syringe containing 150 μl of CBSS and heparin. The single trypanosome was then immediately inoculated into a mouse immunosuppressed with cyclophosphamide 24 hr previously. The mice were monitored daily for the presence of trypanosomes for up to 14 days. The parasites were then harvested at high parasitaemia (greater than 3.2×10^7 ml^{-1} of blood) for PCR analysis. 200 μl of infected blood was used to make stabilates on the same day as harvesting from the mouse and cryopreserved (See below). The remainder of the blood was differentially spun at 1,100 g for 7 mins to separate the trypanosomes from the blood. The serum was decanted and the buffy coat was removed and used to make DNA preparations for PCR analysis.

2.2.4 *In vitro* growth of procyclic form trypanosomes

Blood was taken by cardiac puncture from infected ICR mice. Ten drops of infected blood was added to a culture flask containing 4.5 ml SDM-79 with 10% foetal calf serum and 3mM cis–Aconitate (to transform to procyclics forms) under sterile conditions (Brun and Schonenberger, 1981; Hunt *et al.*, 1994) and placed at 27°C for two-three days. The culture medium, containing procyclic forms was decanted, leaving the blood at the bottom of the flask, and re-suspended in 5 ml SDM-79 with 10% foetal calf serum and left to grow to a trypanosome density of 1 x 10^6 / ml at 27°C.

2.2.5 Procyclic cloning

Procyclic trypanosomes were cultivated in SDM-79 with 10% foetal calf serum (Gibco) and 2% gentamycin (Sigma) (Brun and Schonenberger, 1979) (See Appendix Table A1.A). A 10 µl aliquot was taken from the culture flask and the trypanosomes were counted using an Improved Neubauer haemocytometer. Procyclic clones were then obtained by diluting the culture to a concentration of 10 trypanosomes/ml in Cunninghams medium supplemented with 30% foetal calf serum and 2% streptomycin/penicillin. Cunninghams medium contains supplementary components to SDM–79 and has been shown to promote the growth of single trypanosomes, see Appendix Table A1.B (Cunningham, 1977). 100 µl aliquots of the diluted trypanosomes were added to the wells of a 96 well plate so as to obtain an average density of one trypanosome per well. The plate was then incubated at 27°C in an atmosphere of 5% CO_2 for 14–28 days. The wells were screened for growing trypanosomes by microscopy and positive wells expanded by transferring the trypanosomes to culture flasks

containing SDM – 79 and 10% foetal calf serum. After 2 – 3 days of growth DNA was prepared and the trypanosome genotype determined.

2.2.6 Stabilate preparation of bloodstream forms

Blood was withdrawn from an infected mouse and an equal volume of 7.5 % DMSO in CBSS (final concentration) was added to the blood. This was then injected into a 30 cm length of 2 mm Portex tubing (Portex Ltd) and the tubing cut into 1 cm lengths. These were then placed horizontally into a cryotube, placed in a polystyrene box filled with cotton wool and left at –70°C for 4–6 hr. The stabilates were then transferred to a liquid nitrogen container until required.

2.2.7 Stabilate preparation of procyclic forms

Cells at a density of not less than 10^6 / ml were taken from a culture flask and 900 μl of cell suspension was placed in a cryotube and 100 μl glycerol was added and the contents mixed by gentle inversion. The tubes were then placed at –70°C in a polystyrene box (described above), left overnight, and then transferred to liquid nitrogen until required.

2.2.8 General Polymerase chain reaction (PCR) procedure

All clones generated from both bloodstream and procyclic forms were genotyped using the polymorphic markers CRAM, MS 45, 292, PLC and JS2 (MacLeod *et al.*, 1999; Barrett *et al.*, 1997; Sasse *et al.*, 1998; MacLeod, 1999). The primer sequences for these polymorphic markers are listed in Table 2.3. Due to the sensitivity of PCR, every precaution was taken to ensure that all reagents and materials were kept free of

contaminating DNA. Therefore, all pipette tips and tubes were taken directly from the manufacture's packaging in order to minimize exposure to laboratory contaminants. All PCR preparation was carried out in a laminar flow hood to avoid aerosol contamination.

All PCR reactions were prepared in 10 μl reaction volumes, unless otherwise stated, in 45mM Tris-HCl pH 8.8, 11mM $(NH_4)_2SO_4$, 4.5 mM $MgCl_2$, 6.7mM 2-mercaptoethanol, 4.4mM EDTA, 113 μg/ml BSA, 1mM each of the four deoxyribonucleotide triphosphates, 1 μM of each oligonucleotide primer and one unit of Amplitaq Polymerase (Perkin Elmer, Cetus USA) using as the DNA template 1 μl of diluted crude lysate. Reaction mixtures were overlaid with mineral oil to prevent evaporation and amplifications were carried out in a Robocycler gradient 96 (Stratagene). The cycling conditions for each primer are listed in Table 2.3. PCR products were separated by electrophoresis in a 1 % Seakem agarose gel in 0.5 x TBE buffer (See Appendix A1) or a 4 % NuSieve gel for products under 800 bp and stained with ethidium bromide (0.5 μg/ml) and visualized by UV illumination.

Primer	5´ - 3´ sequence	PCR Conditions	Gel Type	Genotype 247	Genotype 386	Genotype 927
CRAM – G	ctgctgatgccgtacatgatgatttc	96°C 64°C 70°C 50 sec 50 sec 3 min x 28 cycles	1 % Seakem	1 - 1	1 - 2	3 - 4
CRAM – H	aactccctcccgatcgatcacaac					
292 – G	acacccctctccacttcagatac	96°C 64°C 70°C 50 sec 50 sec 3 min x 28 cycles	1 % Seakem	5 - 5	1 - 2	3 - 4
292 – H	gctgaacctgtgggcccctcaattg					
MS42 – F	ttgtgcggtccttaacgcgcgttcaa	96°C 64°C 70°C 50 sec 50 sec 3 min x 28cycles	1 % Seakem	5 - 5	1 - 2	3 - 4
MS42 – W	ggtga-tcatcggctccctttacca					
JS2 – A	gattggcgcaacaactttcacatacg	96°C 56°C 66°C 50 sec 50 sec 50 sec x 30 cycles	4 % NuSieve	5 - 6	1 - 2	3 - 4
JS2 – B	ccctttcttccttggccattgtttttactat					
PLC – G	caacgacgttggaagagtgtgaac	95°C 58°C 66°C 50 sec 50 sec 50 sec x 28 cycles	3 % NuSieve	5 - 5	1 - 2	3 - 4
PLC – H3	ccactgacctttcatttgatcgctttc					

Table 2.3: Oligonucleotide primers used for the determination of unique hybrid progeny derived from STIB 247 x STIB 386 and STIB 247 x TREU 927 crosses. Columns 1 and 2 give the primer name and sequence, respectively. Column 3 lists the PCR conditions for each set of primers. The gel type required for imaging the PCR products is listed in column 4. Finally, column 5 gives the scoring of alleles for each of the three parental lines used throughout this study.

53

2.2.9 Quality control analysis for the determination of unique genotypes

Any possible new unique F1 progeny found were re–genotyped and scored by A. MacLeod and A. Tweedie independently. Both sets of scored genotypes were then given to A. Tait for comparison to make sure they were new unique hybrids before any further analysis was carried out. A triple checking system is critical for linkage analysis, making sure all hybrids are unique and scored correctly.

The difference between independent progeny clones and unique progeny clones should be made clear. An independent progeny clone is defined as either having an unique genotype from all other progeny clones derived from the same fly when genotyped with the five polymorphic markers described above or, if not of unique genotype, then isolated from a different mating event i.e. a different fly.

2.3 RESULTS

Genetic characterisation of clones as being hybrid was based on the inheritance of two PCR amplified bands that corresponded to one band from each parent for all the markers used. With the five polymorphic markers used for genotyping the progeny there were 64 possible progeny genotypes, which could be generated from each of the crosses STIB 247 x TREU 927 and STIB 247 x STIB 386. This is because STIB 247 was homozygous for each of four of the markers CRAM, MS42, 292 and PLC whereas STIB 386 and TREU 927 were both heterozygous for each marker. All three parents were heterozygous for the microsatellite JS2 (Fig 2.2 A – E). This prediction is based on the assumption of a Mendelian system of inheritance, i.e. the progeny are the products of mating between the two parental stocks, and that each inherits one allele from each parent.
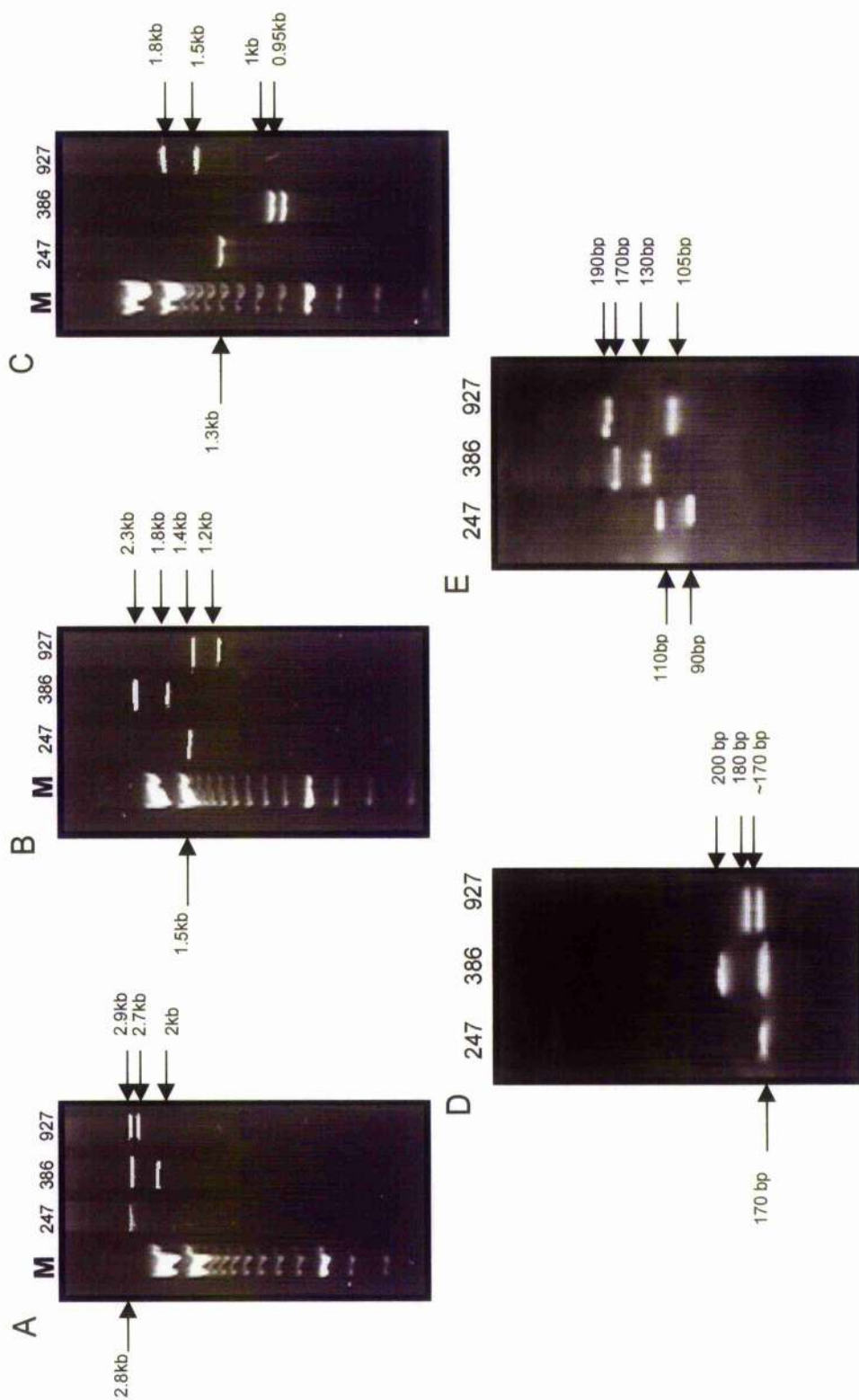
Fig 2.2 A-E Amplification by PCR of mini and microsatellite loci from the three stocks STIB 247, STIB 386 and TREU 927. (A) CRAM, **(B)** MS42, **(C)** 292, **(D)** PLC and **(E)** JS2 from DNA isolated from the stocks STIB 247, STIB 386 and TREU 927 using primer pairs listed in Table 2.2 and PCR conditions described in Materials and Methods. **M** is a 100 bp DNA marker.

2.3.1 Generation of unique F1 hybrid progeny *in vitro*

One stabilate containing the uncloned population F532/72 was thawed, inoculated into an ICR mouse and infected blood was collected and the trypanosomes transformed into procyclic stage trypanosomes (See Materials and Methods, section 2.2.4). Single procyclic trypanosomes were isolated from this culture and grown as clones. Initially cloning was carried out using SDM–79 medium, but no growing trypanosomes were obtained. Cunningham's Medium contains supplementary components that have previously been shown to promote the growth of single trypanosomes (Cunningham, 1977) and so this medium was tested in the cloning procedure and proved to be successful. Over 5,000 wells were screened for the presence of growing trypanosomes. After screening these wells for 4–5 weeks, a relatively low proportion of the wells were positive for growing trypanosomes, with 450 wells in total (9 %). It was found that 137 of the 450 wells (30 %) contained 'clones' that had inherited all alleles from both parents for one or more of the markers (See Appendix Table A2.A) and were thus likely to contain mixtures of more than one genotype (discussed later in this chapter). A further five clones had parental genotypes, that is they only inherited the two alleles of TREU 927, this is discussed later in the chapter. The remaining 308 clones were identified as F1 progeny, however the majority of these were identical to each other in terms of genotype using the markers described. For example, 52 F1 progeny generated from the same batch of cloning gave identical genotypes for the markers used therefore only 1 genotype was obtained (Fig 2.3), this occurred for two other batches of clones. One possible explanation for this finding was that selection of a particular genotype occurred either in the mouse or procyclic population prior to cloning. In total only 14 of the 308

(4.5 %) F1 progeny were different genotypes and only 4 of these were new unique genotypes (28%) (Table 2.4). Therefore of the original 450 clones only 0.8 % were new genotypically unique F1 hybrid progeny. All hybrid progeny generated are listed in Table A2.A of the appendix.

Together, the low efficiency rates and the time required for the growth of cloned procyclics (approximately 4 weeks) led me to abandon this method as being too inefficient to generate the numbers of progeny required for this study in the time available. Also, although procyclic clones were useful for genetic mapping and phenotype studies in which the phenotype was expressed in procyclics, they could not be used in the future to study phenotypes expressed only in bloodstream forms, for example anaemia, without cyclical transmission of each new clone through tsetse flies. For these reasons, an alternative approach of optical cloning of bloodstream forms was undertaken.

Fig 2.3: PCR amplification of minisatellite marker 292 for 11 procyclic clones. Lanes 1 – 11 show the same pattern of allele inheritance for all the clones isolated from F532/72, derived from STIB 247 x TREU 927 cross. Hybrids with identical genotype patterns are likely to be vegetative derivatives of a single mating event. **M**, 100bp DNA ladder.

2.3.2 Isolation of unique F1 hybrid progeny *in vivo*

Clones were made from three different uncloned hybrid trypanosome stocks (F124/28, F532/72 and F974/78) from the STIB 247 x TREU 927 cross. The stabilates from the three stocks were thawed, grown in mice and then single trypanosomes isolated optically and regrown in mice as outlined in Materials and Methods. In total, 145 mice were inoculated with single trypanosomes. Of the 145 injected mice, 61 developed trypanosome infections (42 %). After screening of these 61 clones it was found that 31 were F1 progeny (51 %). From the 31 F1 hybrid progeny 15 were new unique F1 progeny (49 %) (Table 2.4). There was some variation between the numbers of F1 progeny generated from each of the three uncloned stabilates populations, with clones from F124/28 producing the highest number of unique hybrids (Table 2.4 and Appendix Table A2.B).

59

Five of the clones derived from mixed infected tsetse flies (one from F124/28 and four from F974/78) demonstrated the same allelic pattern as one of the parents for all of the markers analysed Fig 2.4, also Appendix Table A2.B. This is predicted as it has been shown that sexual recombination is not obligatory (Turner *et al.*, 1990).

The remaining 25 clones (bloodstream forms) were mixtures/trisomic/ triploid. Analysis of these clones using the mini- and microsatellite markers indicated that these clones had inherited three alleles for one or more markers; both alleles from one parent (TREU 927) and one alle from the other (STIB 247) (Appendix Table A2.B). Therefore, they appeard to be trisomic for one or two of the loci examined and appear to have the normal number of alleles for the other mini- and microsatellite markers. Inorder to determine if these clones are mixtures or trisomic they would require re–cloning. If the three band pattern disappeared on re–cloning then this would suggest that the clone was a mixture of two hybrid clones which differed at one/two loci. If, however, the clones continue to give a three band pattern after re–cloning this would indicate that these clones carried an extra allele at the locus/loci and it could be concluded that these clones were trisomic. Only one batch of clones (procyclic forms) appeared to be triploid, the results indicate that clones F532/72 pcl 1-118 are probably trisomic for each of the chromosomes tested (each marker is located on a different chromosome), however analysis of their DNA content would need to be determined in order to prove if they are triploid (Appendix Table A2.A). Unfortunately it was beyond the scope of this study to investigate whether the clones were mixtures/trisomic or triploid.

The same procedure was carried out for the generation of F1 progeny from the STIB 247 x STIB 386 cross, using five uncloned populations; F9/41, F19/31, F28/46, F29/46 and F57/40. In total 140 mice were inoculated with single trypanosomes from the five uncloned stocks and 44 developed trypanosome infections (31%). After screening with five mini and microsatellite markers it was found that three of the clones were parental (had the same genotypic pattern as the 386 parent for all markers) and 2 were mixtures/trisomic/triploid. The remaining 39 clones were F1 hybrid clones of which 22 were new unique genotypes (56 %) (Table 2.4 and Appendix Table A2.C). Similar numbers of unique hybrids were derived from each of the populations used. However for stabilate F57/40, only 3 clones were produced and when genotyped it was found that they were identical to the STIB 386 parent.

Fig 2.4: Identification of parental clones derived from a genetic cross of STIB 247 x TREU 927. Lanes 1 – 7 are bloodstream clones derived from the optical cloning of F974/74. Genotype analysis with the minisatellite 292 revealed that clones 1, 2, 4 and 5 had alleles of the parental line TREU 927 and so were considered parental clones. **M** 100bp DNA ladder.

Some hybrids inherited more than two alleles from the parental lines when analysed with one of the mini or microsatellite markers. However, for the other markers the same clones appeared to have inherited two alleles only. Fig 2.5 illustrates tri–allelic inheritance for the minisatellite marker 292. There are three plausible explanations for this result. Firstly, that the sample was a mix of two hybrid clones that differed only at the 292 locus. Secondly, the clone could be trisomic for the chromosome carrying the 292 marker (chromosome III). Finally, a gene duplication event could have generated an extra copy of the locus (MacLeod, A., personal communication). In order to prove if the clones displaying a three band pattern were trisomic they would all have needed to be recloned and their genotype determined. If the three band pattern was still observed then the clones would be considered trisomic. If the clones, however, no longer gave a three band pattern after recloning then the original stock must have been a mixture of two hybrid clones. Unfortunately it was beyond the scope of this study to reclone all the possible trisomic clones. Only one mix/trisomic clone was recloned to determine if it was trisomic and also to potentially generate more unique F1 progeny from a mixture. The results of the recloning of F532/72 bscl 15 are discussed below.
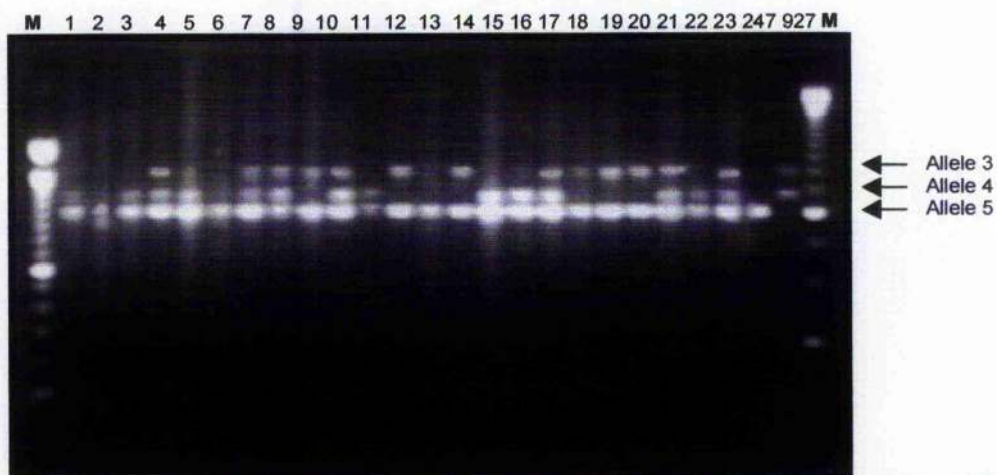
Fig 2.5: Identification of mixtures/trisomic clones. Lanes 1 – 23 were bloodstream clones isolated after optical cloning of F124/28 derived from STIB 247 x TREU 927 cross. Lanes 4, 7, 8, 10, 21 and 23 have inherited two alleles from parent TREU 927 giving a 3 banded pattern when genotyped with the minisatellite 292. Further analysis would be required to determine if these clones are trisomic or just mixtures (See text). **M,** 100bp and 250bp DNA ladders. All PCR conditions are listed in Materials and Methods and Table 2.2

It is obvious from these data that there is a startling difference between the numbers of new unique F1 hybrid progeny generated from bloodstream compared to procyclic cloning approaches. Analysis of all the clones using the four minisatellites and one microsatellite are presented in Tables A2.A–C of the appendix and a summary of the results is presented in Table 2.5. From the procyclic cloning only 1.3 % of the 308 F1 hybrid clones were new unique F1 progeny whereas with the optical cloning of bloodstream forms 43 % and 56 % of the F1 clones were new for the STIB 247 x TREU 927 and STIB 247 x STIB 386 crosses, respectively. The only limitations with generating even larger numbers of bloodstream form clones were the numbers of animals required and the time and labour involved.

In order to try and boost the number of hybrids for the F532/72 population a mix/trisomic clone previously isolated from the first round of cloning, F532/72 bscl 15, was recloned. This isolate had a mix of parental alleles for marker JS2, that is, it had inherited more than one allele from one of the parents. After recloning of F532/72 bscl 15, the three band pattern for JS2 disappeared, confirming that the original stock must have been a mixture of two hybrid clones. Analysis revealed one further new unique hybrid, F532/72 bscl15 5b, from the seven sub-clones that were isolated (Appendix Table A2.B).

There may be several explanations for the variation observed in the numbers of unique hybrid progeny isolated from the different crosses. For example, only 3 new genotypes were detected from F532/72 bloodstream clones in comparison to 10 for F124/28 from the STIB 247 x TREU 927 crosses. However for the STIB 247 x STIB 386 cross there were approximately equal numbers of unique hybrid progeny generated from each of the uncloned stabilates. There are several proposed explanations for the lack of genetic diversity amongst the hybrids generated from F532/72. 1) It is possible that selection for particular genotypes occurs in the salivary glands of the tsetse, with certain genotypes not being viable. 2) The expansion of the population in a mouse selected for a particular genotype. 3) It has been shown that the parental stocks have different rates of growth in mice (Turner *et al.*, 1995). The most likely scenario is, however, that as bloodstream clones had previously been isolated from fly F532/72, the predominant genotypes had already been identified.

In summary, the results of these experiments were that the number of progeny available for genetic analysis for the cross STIB 247 x TREU 927 increased from 18 to 38. The equivalent increase for the cross STIB 247 x STIB 386 was from 12 to 34. The number of hybrid progeny aids in the determination of Mendelian inheritance of the trait of interest i.e. if it segregates in a 1:1 ratio. Therefore, if the number of hybrid progeny is high then proving or disproving Mendelian inheritance is much easier and gives confidence in the result determined.

Table 2.4: All genotypically unique F1 hybrid progeny generated from genetic crosses.

Genotypically unique trypanosome F1 hybrids derived from genetic crosses

Identification	Lysate No.	GUP No.	Markers				
			CRAM	292	MS42	JS2	PLC
Parental 247			1 - 1	5 - 5	5 - 5	5 - 6	5 - 5
Parental 386			1 - 2	1 - 2	1 - 2	1 - 2	1 - 2
Parental 927			3 - 4	3 - 4	3 - 4	3 - 4	3 - 4
Cross 247 x 927							
F124/28 bscl C5	71	4635	1 - 4	3 - 5	4 - 5	4 - 6	4 - 5
F124/28 bscl B3	73	4923	1 - 4	3 - 5	4 - 5	4 - 6	3 - 5
F974/70 mcl 4	77	3086	1 - 4	3 - 5	4 - 5	3 - 5	4 - 5
F532/63 bscl 2	80	4630	1 - 4	3 - 5	3 - 5	3 - 6	4 - 5
F532/63 bscl 3	78	4925	1 - 4	4 - 5	4 - 5	4 - 5	4 - 5
F532/63 bscl 7	79b	4631	1 - 4	4 - 5	4 - 5	3 - 6	4 - 5
F532/63 bscl 5	81	4634	1 - 3	3 - 5	4 - 5	3 - 6	4 - 5
F532/72 mcl 5	88	4392	1 - 4	4 - 5	4 - 5	3 - 5	3 - 5
F532/72 mcl 1	84	4391	1 - 4	4 - 5	3 - 5	3 - 5	3 - 5
F532/72 mcl 2	85	4636	1 - 4	4 - 5	3 - 5	4 - 5	4 - 5
F532/72 mcl 3	86	4427	1 - 4	4 - 5	4 - 5	4 - 5	3 - 5
F532/72 mcl 6	89	4393	1 - 3	3 - 5	3 - 5	4 - 5	4 - 5
F532/72 mcl 7	90	4628	1 - 4	4 - 5	4 - 5	4 - 6	3 - 5
F532/72 mcl 8	91	3538	1 - 4	3 - 5	4 - 5	4 - 5	3 - 5
F532/63 cl 16	171		1 - 4	3 - 5	4 - 5	3 - 5	3 - 5
F532/72 mcl 9	92	4366	1 - 4	4 - 5	4 - 5	3 - 6	3 - 5
F532/63 cl A11	170		1 - 3	4 - 5	3 - 5	3 - 6	4 - 5
F532/63 bscl A14/1	346		1 - 3	3 - 5	4 - 5	3 - 6	3 - 5
F532/72 pcl 1	334		1 - 3	4 - 5	4 - 5	3 - 5	4 - 5
F532/72 pcl 5	359		1 - 3	4 - 5	3 - 5	4 - 5	3 - 5
F532/72 pcl 7	362		1 - 4	3 - 5	3 - 5	4 - 6	3 - 5
F532/72 pcl 8	374		1 - 3	4 - 5	3 - 5	4 - 6	F
F532/72 bscl 1	356		1 - 4	3 - 5	3 - 5	3 - 5	3 - 5
F532/72 bscl 2	357		1 - 4	3 - 5	3 - 5	4 - 5	4 - 5
F532/72 bscl 15/5B	434		1 - 3	3 - 5	3 - 5	4 - 6	3 - 5
F124/28 bscl 1	361		1 - 3	4 - 5	3 - 5	4 - 6	3 - 5
F124/28 bscl 3	368		1 - 3	3 - 5	4 - 5	4 - 5	4 - 5
F124/28 bscl 5	369		1 - 4	3 - 5	4 - 5	3 - 5	4 - 5
F124/28 bscl 9	363		1 - 4	3 - 5	3 - 5	3 - 6	3 - 5
F124/28 bscl 12	364		1 - 4	3 - 5	3 - 5	4 - 6	3 - 5

Table 2.4 cont.

Genotypically unique trypanosome F1 hybrids derived from genetic crosses							
			Markers				
Identification	Lysate No.	GUP No.	CRAM	292	MS42	JS2	PLC
Parental STIB 247			1 - 1	5 - 5	5 - 5	5 - 6	5 - 5
Parental STIB 386			1 - 2	1 - 2	1 - 2	1 - 2	1 - 2
Parental TREU 927			3 - 4	3 - 4	3 - 4	3 - 4	3 - 4
Cross 247 x 927							
F124/28 bscl 13	365		1 - 3	3 - 5	3 - 5	4 - 6	3 - 5
F124/28 bscl 14	375		1 - 4	3 - 5	4 - 5	3 - 5	3 - 5
F124/28 bscl 15	366		1 - 3	4 - 5	4 - 5	4 - 5	4 - 5
F124/28 bscl 20	367		1 - 3	3 - 5	3 - 5	4 - 5	F
F124/28 bscl 22	373		1 - 4	4 - 5	4 - 5	4 - 5	4 - 5
F974/78 bscl 3	370		1 - 4	4 - 5	3 - 5	4 - 5	4 - 5
F974/78 bscl 6	371		1 - 3	4 - 5	4 - 5	4 - 6	3 - 5
F974/78 bscl 7	372		1 - 4	3 - 5	3 - 5	4 - 5	4 - 5
Cross 247 x 386							
F9/45 mcl 2	30	3300	1 - 2	1 - 5	2 - 5	1 - 5	1 - 5
F9/45 mcl 10	34	5060	1 - 1	2 - 5	1 - 5	1 - 6	2 - 5
F9/45 mcl 11	35	3287	1 - 2	1 - 5	1 - 5	1 - 5	2 - 5
B8O cl 2	162		1 - 2	2 - 5	2 - 5	2 - 6	2 - 5
F492/50 bscl 1	174	174	1 - 1	2 - 5	1 - 5	2 - 6	2 - 5
F492/50 bscl 5/1b	332	5316	1 - 2	1 - 5	1 - 5	2 - 6	2 - 5
F492/50 bscl 8	181	5317	1 - 1	2 - 5	1 - 5	1 - 6	2 - 5
F492/50 bscl 9	182	4688	1 - 2	2 - 5	2 - 5	2 - 5	2 - 5
F492/50 bscl 12	185		1 - 1	2 - 5	2 - 5	2 - 5	1 - 5
F492/50 bscl 18	191	4949	1 - 2	1 - 5	1 - 5	2 - 6	2 - 5
F492/50 bscl 21	194	4950	1 - 1	1 - 5	1 - 5	2 - 6	2 - 5
F492/50 bscl 23	196	4954	1 - 2	1 - 5	1 - 5	2 - 5	2 - 5
F9/41 bscl 1	415		1 - 1	2 - 5	2 - 5	1 - 6	1 - 5
F9/41 bscl 2	416		1 - 1	1 - 5	1 - 5	1 - 6	1 - 5
F9/41 bscl 5	383		1 - 2	2 - 5	2 - 5	1 - 5	2 - 5
F9/41 bscl 7	384		1 - 2	2 - 5	2 - 5	1 - 5	1 - 5
F9/41 bscl 8	420		1 - 1	2 - 5	1 - 5	1 - 5	1 - 5
F9/41 bscl 9	385		1 - 1	1 - 5	1 - 5	2 - 6	2 - 5

Table 2.4 cont.

Genotypically unique trypanosome F1 hybrids derived from genetic crosses							
			Markers				
Identification	**Lysate No.**	**GUP No.**	**CRAM**	**292**	**MS42**	**JS2**	**PLC**
Parental STIB 247			1 - 1	5 - 5	5 - 5	5 - 6	5 - 5
Parental STIB 386			1 - 2	1 - 2	1 - 2	1 - 2	1 - 2
Parental TREU 927			3 - 4	3 - 4	3 - 4	3 - 4	3 - 4
Cross 247 x 386							
F9/41 bscl 11	422		1 - 2	1 - 5	1 - 5	1 - 5	2 - 5
F19/31 bscl 1	389		1 - 2	2 - 5	1 - 5	1 - 6	1 - 5
F19/31 bscl 5	425		1 - 1	1 - 5	1 - 5	1 - 6	2 - 5
F19/31 bscl 8	428		1 - 2	1 - 5	1 - 5	2 - 5	2 - 5
F19/31 bscl 10	430		1 - 2	1 - 5	1 - 5	2 - 6	2 - 5
F19/31 bscl 11	391		1 - 1	1 - 5	1 - 5	1 - 5	2 - 5
F28/46 bscl 1	407		1 - 1	1 - 5	1 - 5	2 - 5	1 - 5
F28/46 bscl 4	409.1		1 - 2	1 - 5	1 - 5	2 - 6	2 - 5
F28/46 bscl 6	394.1		1 - 2	1 - 5	1 - 5	2 - 5	2 - 5
F28/46 bscl 7	410.1		1 - 1	2 - 5	1 - 5	2 - 5	1 - 5
F28/46 bscl 8	411		1 - 2	2 - 5	2 - 5	2 - 6	2 - 5
F28/46 bscl 11	405		1 - 2	2 - 5	2 - 5	2 - 5	2 - 5
F29/46 bscl 1	414		1 - 1	2 - 5	1 - 5	1 - 5	1 - 5
F29/46 bscl 2	406		1 - 1	1 - 5	1 - 5	2 - 6	2 - 5
F29/46 bscl 3	386.1		1 - 1	1 - 5	1 - 5	2 - 6	2 - 5
F29/46 bscl 4	387.1		1 - 1	1 - 5	1 - 5	2 - 6	1 - 5
Cross 386 x 927							
F296/39 22/1	264		1 - 4	1 - 3	1 - 3	1 - 4	2 - 3
F296/39 24/1	269		1 - 4	2 - 3	1 - 3	2 - 3	1 - 3
F296/39 bscl 7	207		1 - 4	2 - 3	1 - 3	2 - 3	1 - 3
F296/39 bscl 9	209		1 - 4	2 - 3	1 - 3	2 - 3	1 - 3
F296/44 bscl 1	2	3199	2 - 3	2 - 3	1 - 3	1 - 3	2 - 3
F296/44 bscl 3	6	3201	1 - 3	1 - 3	1 - 3	1 - 4	2 - 3
F296/44 bscl 4	5	3204	1 - 4	1 - 4	1 - 4	1 - 3	1 - 3

Table 2.4: All genotypically unique F1 hybrid progeny generated from genetic crosses. The cross from which each clone is derived and individual identification numbers given to each hybrid clone are listed in column 1 and these are used throughout the study. The nomenclature follows that of Sternberg *et al.*, 1989. For example F28/46 bscl 6, was the sixth clone isolated from fly 28, on day 46 post infection. Columns 2 and 3 give additional identification numbers i.e. stabilates and lysate numbers, where available. Columns 4-7 give the results of the mini and microsatellite marker analysis. Alleles are numbered 1-5 for the minisatellites, CRAM, 292, MS42 and PLC, and 1-6 for the microsatellite JS2. Shaded rows indicate the new unique F1 hybrid progeny generated in this study. GUP, Glasgow University Parasitology. Bloodstream clones from the same tsetse fly, sampled on the same day and conferring the same genotypic pattern were considered to have arisen from one sexual recombination event and so were represented by one clone in the above tables. The complete sets of results of all clones are listed in Tables A2.A–C of the Appendix.

Stabilate	BSCL or PCL	P 1	P 2	Total No. of Clones	Hybrid progeny	Mix / Trisomic/Triploid	Parentals	No. Unique progeny
F532/72	PCL	247	927	450	313	137	0	4
F532/72	BSCL	247	927	18	10	8	0	2
F124/28	BSCL	247	927	34	16	17	1	10
F974/78	BSCL	247	927	9	5	0	4	3
F532/72 15/5B	BSCL	247	927	7	7	0	0	1
F9/41	BSCL	247	386	11	10	1	0	7
F19/31	BSCL	247	386	14	13	1	0	5
F28/26	BSCL	247	386	11	11	0	0	6
F29/46	BSCL	247	386	5	5	0	0	4
F57/40	BSCL	247	386	3	0	0	3	0

Table 2.5: Summary table of all clones generated for STIB 247 x TREU 927 and STIB 247 x STIB 386 crosses. Listed in column 1 is the identification of the tsetse fly from which the clones were derived, using the nomenclature previously described by Sternberg *et al.*, 1989. Column 2 states whether the clones were procyclic forms (PCL) or bloodstream forms (BSCL). Columns 3 and 4 are the parental lines used for the genetic cross. Columns 5–8 list the overall total of clones obtained from cloning of single trypanosomes from each tsetse fly, the number of hybrid progeny, number of mixes/trisomics/triploids and parentals. Finally, column 9 lists the overall number of new unique F1 hybrid progeny generated from each tsetse fly. P1 and 2, parent 1 and 2.

2.3.3 Inheritance of mini and microsatellite marker for all hybrids generated from crosses.

The alleles at each locus appear to be inherited in a Mendelian manner as most allele combinations were observed in the F1 hybrid progeny (Table 2.4). The pattern of allele inheritance also shows that alleles at unlinked loci assort independently (see for example Fig 2.6). Thus the progeny are genetically different and as a result must be the products of independent mating events.

For the STIB 247 x TREU 927 cross, 16 unique genotypes were detected out of 38 hybrids (excluding mixes and parentals) from optical cloning of bloodstream forms and of the 308 procyclic clones (excluding mixes) only four had unique genotypes. With the STIB 247 x STIB 386 cross 22 unique genotypes were detected out of 39 hybrids (excluding mixes and parentals) (Table 2.5). As shown in Table 2.4 some genotypes of the unique progeny clones are identical but because they have been isolated from different flies (independent crosses) they were considered as independent progeny as they were products of separate mating events. The probability of obtaining an identical progeny clone from two independent crosses will be very low in the absence of selection. Thus although the progeny clones are identical for the five markers screened here, they should be different when further markers are analysed.

A CRAM

B MS42
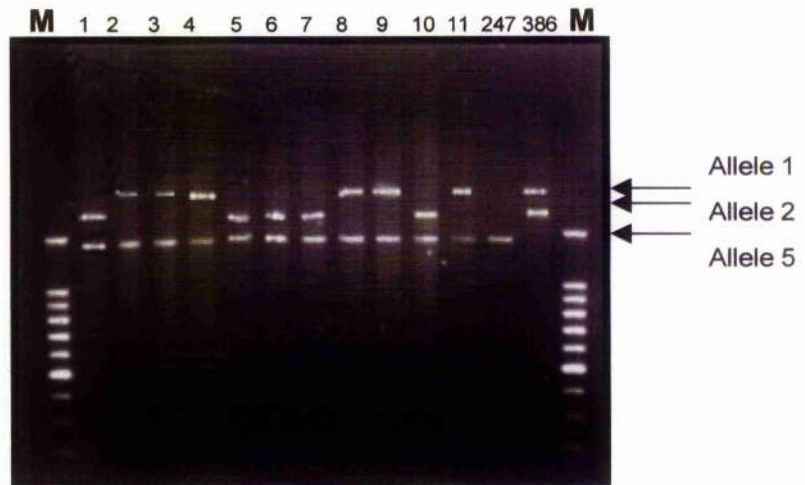
Fig 2.6 Inheritance of minisatellite markers for hybrid clones. STIB 247 x STIB 386 cross hybrid progeny clones (lanes 1 – 11) generated from the population F9/41 screened for (A) CRAM and (B) MS42. Each panel shows allelic segregation and a comparison of the panels shows independent assortment. **M** is a 100 bp DNA marker.

2.3.4 Test for Mendelian inheritance of each marker for STIB 247 x TREU 927 and STIB 247 x STIB 386 crosses

The generation of a larger number of unique progeny clones and their genotyping with five markers each located on different chromosomes allows the testing of whether *T. brucei* has a Mendelian genetic system. In order to determine if the ratio of the hybrid genotypes segregate in a manner that conforms to those predicted by Mendelian segregation a statistical analysis was undertaken (Table 2.6). The data used to determine Mendelian segregation were obtained by combining the results from the clones previously generated (MacLeod, 1999) and the newly generated clones from this study. Comparisons were made between the observed frequency of genotypes and the expected number of genotypes assuming 1:1 segregation of alleles inherited from either STIB 386 or TREU 927. This analysis demonstrated that the majority of markers do not deviate from the allele segregation ratio predicted by Mendelian inheritance i.e. 1:1 ratio. Two markers, MS42 and PLC however, show a statistical deviation from a 1:1 segregation, for the STIB 247 x STIB 386 cross. There are a number of reasons that could explain why these markers did not agree with Mendelian segregation; 1) Segregation distortion where there was selection of one particular genotype over another, 2) There was a relatively small sample size, therefore, if the number of hybrid progeny was increased this distortion could disappear.

Cross	Marker	Segregating Alleles	Observed (Expected)	χ^2	df	Accept/ reject Ho significance of 0.05
STIB247 x TREU927	CRAM	3	15 (19)			Accept
		4	23 (19)	1.68	1	0.2 > P > 0.1
	MS 42	3	19 (19)			Accept
		4	19 (19)	0	1	P < 0.99
	292	3	21 (19)			Accept
		4	17 (19)	0.42	1	0.7 > P > 0.5
	JS2	3	14 (19)			Accept
		4	24 (19)	2.6	1	0.2 > P > 0.1
	PLC	3	19 (19)			Accept
		4	19 (19)	0	1	P < 0.99
STIB247 x STIB386	CRAM	1	17 (17)			Accept
		2	17 (17)	0	1	P < 0.99
	MS 42	1	25 (17)			**Reject**
		2	9 (17)	7.52	1	**P < 0.01**
	292	1	20 (17)			Accept
		2	14 (17)	1.06	1	0.5 > P > 0.3
	JS2	1	14 (17)			Accept
		2	20 (17)	1.06	1	0.5 > P > 0.3
	PLC	1	11 (17)			**Reject**
		2	23 (17)	4.2	1	**0.05 > P > 0.02**

Table 2.6: Statistical test of segregation ratios for each of the five markers in the crosses STIB 247 x TREU 927 and STIB 247 x STIB 386. The numbers of each allele type inherited into the F1 progeny is indicated in column 3 (observed). The expected numbers are shown in brackets and assume a 1:1 segregation in all cases. The final column shows whether or not the null hypothesis is accepted or rejected at a significance level of 0.05.

2.4 DISCUSSION

The generation of new unique F1 hybrid progeny from previously uncloned populations from STIB 247 x TREU 927 and STIB 247 x STIB 386 crosses increased the number available for further analysis from 18 to 38 and 12 to 34 respectively.

There was a marked variation in the number of unique genotypes generated by cloning procyclic forms or bloodstream forms. Only four new progeny clones were generated from procyclic forms whilst all other new F1 hybrids were derived from bloodstream form cloning. The *initial* problem with procyclic cloning was the culture medium used. Whilst SDM-79 was effective for the long term maintenance of procyclic forms it was not suitable for the growth of a single trypanosome, giving cloning efficiency rates of 5–10%. It was found that when the same procedure was carried out using Cunningham's medium, with 30% foetal calf serum, the efficiency rate increased to approximately 30%. Even with this increase in efficiency, however, the number of unique genotypes generated was low in comparison to the number produced by the optical cloning of bloodstream forms.

One possible explanation as to why the procyclic cloning was not efficient in generating many different genotypes could be that the trypanosome population had gone through a potential bottleneck that might reduce the diversity in the uncloned population. The reason for this is that within the mammalian host it is known that trypanosomes of different genotypes grow at varying rates, which could lead to selection (Turner *et al.*, 1995).

Optical cloning of bloodstream forms was not, however, without its drawbacks. It required the use of a large number of animals and was a very time consuming, labour intensive task and yielded a high number of mixed infections due to the difficulty in trying to isolate a single trypanosome and introducing it successfully to an immunosuppressed mouse within a minimal time period. However, the data suggest that, in principle, generating large numbers of unique progeny clones would be possible.

The data show that the alleles at five loci segregate into the F1 progeny in ratios that conform to those predicted by Mendelian segregation i.e. 1:1 ratio, for most of the markers tested, as would be predicted in a standard diploid genetic system involving meiosis. The independent assortment of alleles at different loci has also been demonstrated (Fig 2.6). Taken together, the data establish that the sexual process in *T. brucei* follows a Mendelian system. The formal proof that the genetic system is Mendelian allows the analysis of the segregation of phenotypes that differ between the parental lines and from this the analysis of the genetic basis for such phenotypes.

CHAPTER 3

AN INVESTIGATION OF THE POTENTIAL DEVELOPMENT OF BLOODSTREAM FORMS FROM PROCYCLIC FORMS WITHOUT CYCLICAL TRANSMISSION THROUGH TSETSE FLIES

3.1 INTRODUCTION

Procyclic trypanosomes have been widely used in trypanosome research, largely because of the ease with which they can be grown to substantial numbers in *in vitro* culture (Brun and Jenni, 1985). In the particular context of genetic studies, they are useful for genetic mapping and analysis of the inheritance of traits such as arsenical drug resistance where the trait is expressed in procyclic cells. They cannot be used, however, to investigate the inheritance of traits that are expressed only in the bloodstream stage of the life–cycle such as human serum resistance, virulence and anaemia.

Transformation of trypanosomes from bloodstream to procyclic forms is a tractable and well characterised developmental process in the life–cycle (Matthews and Gull, 1994), in comparison with developmental processes in the rest of the life–cycle, which are only poorly understood (Van den Abbeele *et al.*, 1998) and it has proven extremely difficult to replicate development in the fly in *in vitro* culture (Hirumi *et al.*, 1992).

There would be considerable advantages in being able to move trypanosomes from procyclic forms, which are easy to grow and genetically manipulate, to bloodstream forms that cause disease if it avoided the requirement for full cyclical development

through tsetse flies. There is slight evidence in the literature that an approach to avoid full development through the life–cycle and transform procyclics to bloodstream forms directly should be possible, but this evidence is barely more than anecdotal reports (Cross, 1975; Kaminsky *et al.*, 1987; Hirumi, 1992).

In this Chapter I describe a systematic series of experiments to follow up on these reports by manipulating procyclic forms in a number of simple ways and testing whether they become infective for mice.

3.2 MATERIALS AND METHODS

3.2.1 Inoculation of mice with procyclic forms

Procyclic forms of STIB 247 and TREU 927 were cultivated in SDM–79 with 10% foetal calf serum, as described in Materials and Methods, section 2.2.4, grown to a density of 2×10^6 /ml. The cells were then re–suspended in, Cunninghams medium with 30% foetal calf serum or SDM–79 with 10 % foetal calf serum. Five immunosuppressed mice for each group (Materials and Methods, section 2.2.2) were inoculated via i.p inoculation with 1×10^6 trypanosomes/ml from log phase cultures. Mice were monitored for 14 days for evidence of parasitaemia. The same procedure was also carried out with procyclic cells that had reached stationary phase of growth. Two procyclic clones (PCL) derived from procyclic cloning, F532/72 PCL 8 and F532/72 PCL 12, were also grown to log and stationary phases in both media listed above and mice were inoculated with 1×10^6 trypanosomes/ml and parasitaemia monitored.

3.2.2 Tsetse fly infection

In parallel, the two procyclic clones, STIB 247 and TREU 927 were separately cultivated in both Cunninghams and SDM–79 media to a density of 2×10^6 /ml. Ten tsetse flies (*Glossina morsitans*) for each of the four lines and for both types of media were placed in individual holding tubes. The 8 groups of tsetse flies were membrane–fed with 5 ml horse blood containing 1×10^6 trypanosomes/ml of each culture of trypanosomes. A sample from the horse blood inoculated with procyclic forms was taken to check that the cells had not lysed and after 45min the cells were still found to be viable. Two days later the flies were fed again with infected blood. The fifth day after infection the flies were

given a maintenance feed with horse blood. Eight to ten days after the first infective feed the flies were dissected and the presence of a midgut infection determined. The flies were maintained at 28°C in 75% humidity.

3.2.3 Tsetse fly dissection and mouse inoculation

The tsetse flies were placed on ice for 30 min. Their wings and legs were then removed and their body immobilized with a pin through the thorax. The tsetse flies were dissected by making a dorsal mid – line incision through the abdomen. The gut was removed, placed on a slide with a drop of PBS and examined microscopically for the presence of trypanosomes. The entire contents of any positive gut infections were taken up into a syringe containing 1 ml PBS and injected into immunosuppressed mice. One mouse was used for each infected fly gut. The mice were monitored for parasitaemia for 14 days.

3.2.4 Full Cyclical Development in the Tsetse fly

Tsetse flies were infected in the same way as described above. After maintenance for 28 days each tsetse was probed for the presence of a metacyclic infection. This was achieved by placing each tubed fly on a microscope slide on top of a heating plate. The flies are attracted to the heat and they salivate onto the slide. Microscopic examination of the slide determines the presence of metacyclic trypanosomes. If any infection was detected then the flies were allowed to feed directly on an immunosuppressed mouse and the development of a bloodstream infection was monitored over 14 days.

3.3 RESULTS

STIB 247, TREU 927 and two procyclic clones were grown to log phase in SDM–79, inoculated into five mice but yielded no bloodstream infections in mice. Similarly, the 20 mice inoculated with the lines grown to log phase in Cunninghams medium gave no bloodstream infections after 21 days of monitoring. The same result was observed with the cells that had been grown in both culture media until stationary phase – no bloodstream infections developed after 21 days of monitoring for parasitaemia.

Of the 80 tsetse flies infected with STIB 247, TREU 927 and two procyclic clones, 36 survived past one week. Of these 36 flies 23 contained the forms grown in Cunninghams medium. In total 21 flies had developed midgut infections 16 of which were those grown initially in Cunninghams medium (Table 3.1). The percentage of flies in which midgut infections established was in the range of 33–88% (Table 3.1). The values were consistently higher when the procyclic trypanosomes were grown in Cunninghams medium rather than SDM–79. Immunosuppressed mice were inoculated with the gut contents of the tsetse flies suspended in PBS and their parasitaemias monitored for 14 days. After 14 days, however no bloodstream infections were observed.

Procyclic Culture	No. of surviving flies	No. of flies with mid–gut infections DAY 8 - DAY 10	% of fly gut infections	No. of mice developing parasitaemia
STIB 247 (SDM-79)	5	2/5	40	0
STIB 247 (CUNN)	8	7/8	88	0
TREU 927 (SDM-79)	2	1/2	50	0
TREU 927 (CUNN)	5	3/5	60	0
F532/72 PCL 8 (SDM-79)	3	1/3	33	0
F532/72 PCL 8 (CUNN)	6	4/6	66	0
F532/72 PCL12(SDM-79)	3	1/3	33	0
F532/72 PCL 12(CUNN)	4	2/4	50	0

Table 3.1: Tsetse flies infected with procyclic trypanosome cultures. Column 1 shows the procyclic trypanosome lines used to infect tsetse flies and the culture medium they were cultivated in. Column 2 indicates the number of flies, out of ten, which survived infection. Dissection of the surviving tsetse flies showed that 21 of the flies had trypanosome mid–gut infections on either day 8 or 10 post infection as indicated in column 3. The percentage of fly gut infections for each group is given in column 4. Finally, column 5 shows the number of mice developing bloodstream infection after inoculation with the contents of the infected mid–gut. Cunn – Cunninghams medium.

As the percentage of tsetse flies developing fly gut infection was substantially higher using Cunninghams medium it was decided to repeat the experiment. Due to the fact that the procyclic forms in the previous experiment had initially been transformed from bloodstream forms to procyclic forms in SDM–79 and then transferred to Cunninghams it was thought that SDM–79 could have had an adverse effect on the transmissibility to the tsetse flies. Therefore, bloodstream forms of STIB 247 and TREU 927 were grown up in mice, harvested at the peak of parasitaemia and transferred to Cunninghams medium with 30% foetal calf serum (without cis–Aconitate) and grown to a density of 2 x 10^6 /ml (see Materials and Methods, section 2.2.4). These procyclic forms were then

used to infect 20 tsetse flies, 10 for each line, and after 10 days the flies were dissected and the presence of midgut infection determined. It was found that 15 of the 20 flies had midgut infections (Table 3.2). The content of each midgut was transferred to an immunosuppressed mouse, but after 14 days of monitoring no bloodstream infections were observed.

Procyclic Culture	No. of surviving flies	No. of flies with mid-gut infections DAY 10	% of fly gut Infections	No. of mice developing parasitaemia
STIB 247	9	8/9	89	0
TREU 927	9	7/9	78	0

Table 3.2: Tsetse flies infected with procyclic trypanosome cultures grown in Cunninghams medium. Column 1 shows the procyclic trypanosome lines used to infect tsetse flies and the culture medium they were cultivated in. Column 2 indicates the number of flies, out of ten, which survived infection. Dissection of the surviving tsetse flies showed that 15 of the flies had trypanosome mid-gut infections on either day 10 post infection as indicated in column 3. The percentage of fly gut infections for each group is given in column 4. Finally, column 5 shows the number of mice developing bloodstream infection after inoculation with the contents of the infected mid-gut.

The two F1 procyclic clones, F532/72 PCL 8 and F532/72 PCL 12 were also taken through full cyclical development in the tsetse fly as positive controls. Of the ten flies infected for each clone two of the flies infected with F532/72 PCL 8 had metacyclics present in the salivary glands and three of the flies infected with F532/72 PCL 12. After these five flies were allowed to feed on immunosuppressed mice bloodstream infections were observed in one of the mice inoculated with F532/72 PCL 8 metacyclics and two of the mice that had been inoculated with F532/72 PCL 12 metacyclics. This indicated that full cyclical development in the tsetse fly is required to obtain bloodstream forms from procyclic forms. These two clones are represented in Table 2.4 as F532/72

BCL 1 and BCL 2, respectively as they were transformed from procyclic clones to bloodstream clones, but only after full development in the tsetse fly. Sarah McLelland did the majority of the positive control analysis.

3.4 DISCUSSION

Unfortunately this was not a very fruitful investigation in that no bloodstream forms were derived from procyclic forms without cyclical transmission through tsetse flies. There are several explanations why transformation from procyclic forms to bloodstream forms without firstly developing to metacyclics during cyclical transmission through tsetse flies may occur.

The life cycle stages in the vector are well documented, from the procyclic midgut infection to the development of metacyclic trypanosomes (Vickerman, 1985) and have been described in Chapter 1. Little is known, however, about the intermediate phase that involves the migration of *T. brucei* from the midgut through the proventriculus to the salivary glands. It is possible that this migratory phase is of vital biological significance for the completion of the developmental cycle in the tsetse fly (Van den Abbeele *et al.*, 1998). In order to complete the life–cycle the trypanosome must get through two successive barriers, colonization of the midgut and establishment in the salivary gland both of which are independent of each other (Van den Abbeele *et al.*, 1998). The post mesocyclic migratory stage in the parasite life–cycle is an essential bridge for the established midgut infection and metacyclic development in the salivary glands (Van den Abbeele *et al.*, 1998). Midgut infections, which do not continue development, therefore remain procyclic immature infections for the life of the tsetse fly and are non–infective (Welburn and Maudlin, 1997).

During the developmental cycle the trypanosome is expressing different genes. For example, trypanosomes switch off variable antigen gene expression when bloodstream *T. brucei* transform to procyclic forms because of the change in environment. Trypanosomes at different developmental stages, however, may occupy the same environment, e.g. epimastigote and metacyclic forms in the salivary glands. As a result, it is likely that a predetermined series of events leads to the production of the mature metacyclic infective form (Vickerman, 1985). It is not known if the trypanosome cycle is unidirectional i.e. if stages A → B→ C , can also transform B →A or C → B. Similarly, can any stage be omitted completely and full cyclical development still occur (A → C)? One study found that when procyclic trypanosomes were grown *in vitro* in the presence *Anopheles gambiae* cells, some developed into metacyclic forms infective for mice, and the animals developed non–relasping and fatal infections. Control cultures, however, that had been grown in the absence of *A. gambiae* cells also produced forms that lead to infections in mice but these were weak parasitaemias that did not kill the mice during the 30–day period of observation (Kaminsky *et al.*, 1987).

The above data suggest that either reversion or omission of a life–cycle stage may be possible, but contrast with our understanding as to how trypanosomes protect themselves from immune killing in the mammalian host. Bloodstream trypanosomes express variant surface glycoprotein but on entering the fly VSG synthesis is repressed until the salivary gland trypomastigotes reactivate VSG genes and the trypanosomes reacquire their surface coat. It has been shown that the loss of the surface coat is 2 – 3 times faster in the posterior than the anterior midgut, the former being the more

important site for initiation of fly infection (Vickerman *et al.*, 1988). It is this surface coat that enables the trypanosome to evade the hosts' immune responses. It follows, therefore, that as the procyclic forms had no surface coat the immune response mounted by the immunosuppressed mouse was sufficient to kill the trypanosomes before a bloodstream infection developed.

In view of these considerations, anecdotal evidence of the development of bloodstream forms from procyclic forms without complete cyclical transmission through tsetse flies is surprising, but published. This systematic investigation, however, failed to support these published findings.

One interesting finding, however, has been observed from the results obtained. Of the 36 flies that survived infection, 23 had been infected with procyclic forms grown in Cunningham's medium. Also 16 of the 21 flies with midgut infections were cultivated in Cunningham's medium (Table 3.1). These results follow those found previously where Cunningham's medium was more efficient at producing procyclic clones than SDM–79 (Chapter 2). Therefore, in the future, any procyclic trypanosome lines that are to undergo cyclical transmission in the tsetse fly should be cultivated in Cunningham's medium rather that SDM–79 in order to increase the efficiency rates of producing midgut infections.

CHAPTER 4

THE INHERITANCE OF ARSENICAL DRUG RESISTANCE IN GENETIC CROSSES

4.1 INTRODUCTION

Classical genetic analysis can be used to determine the genetic basis of variation of phenotype. This approach exploits a naturally occurring variation in cymelarsan sensitivity between the parasite stocks. STIB 247 is sensitive to killing by the arsenical drug Cymelarsan relative to STIB 386 and TREU 927 (Table 1.1). Genetic crosses between these stocks then permitted a determination of the genetic basis (number of loci, dominance etc) of drug resistance.

This chapter describes the characterisation of the drug resistant phenotype in the three parental stocks and the inheritance into hybrid progeny generated from crosses (Fig 2.1). Currently only the F1 progeny of crosses are available as no F2 or backcross progeny have been generated in sufficient numbers for analysis. Two methods to assess the activity of two classes of trypanocides, the arsenicals and the diamidines have been used; the growth inhibition assay (Kaminsky and Zweygarth, 1989) and the AlamarBlue assay (Raz *et al.*, 1997).

4.2 MATERIALS AND METHODS

4.2.1 Drugs

Cymelarsan (MelCy) was a kind gift from Rhone Merieux, France; Melarsoprol (MelB, Arsobal), Trimelarsan (MelW) and Melarsen oxide (MelOx) were kind gifts from Dr. Frank Jennings, Glasgow University. Pentamidine, Diminazene aceturate (Berenil) and Phenylarsine oxide (PhenOx) were supplied by Sigma. MelCy, MelW, Berenil, Pentamidine and PhenOx were dissolved in phosphate buffered saline (PBS). MelB and MelOx were in an aqueous solution of propylene glycol, at a concentration of 3.6%, and were re–suspended in peanut oil (Sigma) (propylene glycol is miscible in water) immediately prior to use. For the *in vitro* assays, the drugs were dissolved in SDM–79 complete medium. For the *in vivo* assays MelCy was dissolved in double distilled de–ionised water (dddH$_2$O).

4.2.2 *In vitro* 24-hr Growth Inhibition Assay

Samples taken from trypanosome procyclic stage cultures were in log phase of growth. Cymelarsan was dissolved in complete SDM–79 medium and filter-sterilised through a 0.22 μm filter immediately before use. Trypanosome procyclic cultures were resuspended at a density of 2×10^6 /ml in SDM–79 with 10% foetal calf serum. 50 μl of the culture was added to each well of a 96 well microtitre plate containing 50 μl of the drug solutions giving a starting density of 10^6 trypanosomes/ml (Scott *et al.*, 1996). Control cultures were included using the same conditions but with PBS in place of drug. The cultures were incubated at 27°C for 24 hr. All experiments were carried out in duplicate. After incubation, an aliquot of 10 μl was removed from each well and transferred to an Improved Neubauer haemocytometer and the number of cells counted

for each concentration of drug used. The increased number of trypanosomes, which occurred in drug–treated and control cultures, was calculated from each well (number of trypanosomes after 24 hr growth–number of trypanosomes at 0hr) and the relative growth of trypanosome populations was determined by comparison with the number of trypanosomes (100%) occurring in the control cultures. The effective concentration that inhibited the growth of trypanosome populations by 50% (EC_{50}) was determined (values >100% and <0% were rejected) (Kaminsky and Zweygarth, 1989).

4.2.3 *In vivo* drug susceptibility assay

Female inbred ICR mice, weighing 20–25 g, were used for the *in vivo* drug tests based on a method previously described (Turner *et al.*, 1988). Mice were inoculated intraperitoneally (ip) each with 1×10^6 trypanosomes, and treatment was administered 24 hr after inoculation. Drugs were administered ip at the appropriate concentration, at a volume/weight (v/w) ratio of 0.25 ml/25 g of mouse weight. The range of concentrations of MelCy used was 0.1, 0.2, 0.5, 1, 2, 5, and 7.5 mg/kg. The tail blood of the mice was examined three times a week for the presence of trypanosomes for a total of 14 days using the wet film technique. The lowest dose of MelCy that prevented parasitaemia occurring was used to define the sensitivity of each trypanosome line.

4.2.4 *In vitro* growth of trypanosomes for assessment of drug susceptibility for procyclic forms

For the growth of sufficient trypanosomes for the drug sensitivity assay, trypanosomes were taken, by cardiac puncture, from infected ICR mice. Ten drops of infected blood was added to 4.5 ml SDM–79 and 0.5 mls of 3mM cis–Aconitate (to transform to procyclics forms) (Hunt *et al.*, 1994) and placed at 27°C for two-three days. The culture medium, containing procyclic forms was decanted off, leaving the blood at the bottom of the flask, and re-suspended in 5 ml SDM–79, incubated at 27°C and grown to a trypanosome density of 1×10^6.

4.2.5 *In vitro* growth of trypanosomes for assessment of drug susceptibility for bloodstream forms

The medium used for the propagation of bloodstream forms was HMI–9 (Chapter 2) supplemented with 20% foetal calf serum. The same method for the isolation of trypanosomes was used as that for procyclic forms with the exception that no cis–Aconitate was added to the culture medium and the trypanosomes were incubated at 37°C in 5% CO_2/95% air. After 24 hr incubation at 37°C the bloodstream trypanosomes were resuspended at a density of 2×10^6 in HMI–9 ready for analysis of drug sensitivity with the AlamarBlue assay.

4.2.6 AlamarBlue assay

Procyclic or bloodstream stage trypanosomes were suspended at a density of 2×10^6. Drugs were dissolved in SDM–79 and HMI–9, respectively. 100 μl of the cultures were added to each well of a 96 well microtitre plate containing 100 μl of the drug solutions giving a starting density of 10^6 trypanosomes/ml. Control cultures were included using the same conditions but with PBS in place of drug. Finally 20 μl of AlamarBlue dye was added and the plates incubated at 27°C for procyclic forms and 37°C for bloodstream forms for 24hrs. Reduction of AlamarBlue to a fluorescent product only occurs if there are viable cells present. Thus the level of fluorescence, measured at OD_{590}, is proportional to the number of viable cells (Raz *et al.*, 1997). Four arsenical drugs were tested: MelCy, MelW, MelB and melarsen oxide at final drug concentrations of, 0.001, 0.01, 0.1, 1, 10, 100 and 1000 μM as well as phenylarsenoxide at a concentration range of 0.001, 0.01, 0.1 and 1 μM. The sensitivity of the three stocks STIB 247, STIB 386 and TREU 927, to each concentration was assessed. The assay was also carried out using the diamidines; berenil and pentamidine in the same concentration range as MelCy. Each experiment included a control sample containing trypanosomes in the relevant culture media. For testing Pentamidine, cells were incubated in the presence of drug for 24hr, then the dye was added and the cells incubated for a further 24 hr. Fluorescence was measured after 48hr. All assays were repeated at least twice.

4.3 RESULTS

Detailed laboratory based studies on drug resistance have been largely restricted to experiments using mice. The maintenance of trypanosomes *in vitro* is a major step forward in facilitating detailed studies of mechanisms of drug resistance in parasites (Kaminsky *et al.*, 1989). The development of semi–defined medium has been invaluable for this study due to the large number of progeny requiring analysis for their responses to seven different trypanocidal drugs. However given the number of progeny requiring analysis, a rapid, reliable and reproducible screening assay was required. A comparison between two *in vitro* screening assays was carried out to determine which was more suited for this study, the growth inhibition assay or the AlamarBlue assay.

The principal of the growth inhibition assay was based on that previously described (Kaminsky and Zweygarth 1989). Below a certain drug concentration there was no measurable effect on growth (100% growth). Conversely, above a certain concentration of drug no measurable growth could be observed (0% growth). These cut–off points allowed for the calculation of the effective concentration that inhibited 50% growth (EC_{50}). Procyclic forms of the parental lines STIB 247, STIB 386 and TREU 927 were assessed, in duplicate, for their levels of resistance to MelCy. The growth inhibition of the three parental lines after 24 hr incubation in the presence of increasing concentration of MelCy is illustrated in Fig 4.1, where the percentage growth is measured for each line relative to control cultures in the absence of drug.

Growth of STIB 247 was inhibited after incubation with MelCy at a concentration of 0.1 µM and above. No growth was detectable at a concentration of 1 µM. In contrast, STIB 386 and TREU 927 showed only a minor reduction in rate of growth at a concentration of 1 µM MelCy, however the rate of growth diminished at concentrations of 5 µM and above. These data implied that lines STIB 386 and TREU 927 expressed a resistance phenotype in comparison to line STIB 247. Hence, lines STIB 386 and TREU 927 were MelCy resistant and STIB 247 was sensitive. The EC_{50} values were 0.7 µM, 7.5 µM and 8 µM for the lines STIB 247, STIB 386 and TREU 927, for procyclic forms respectively (Fig 4.1 A). For the bloodstream forms the EC_{50} were 0.3 µM, 5 µM and 6 µM for the lines STIB 247, STIB 386 and TREU 927, respectively (Fig 4.1 B)
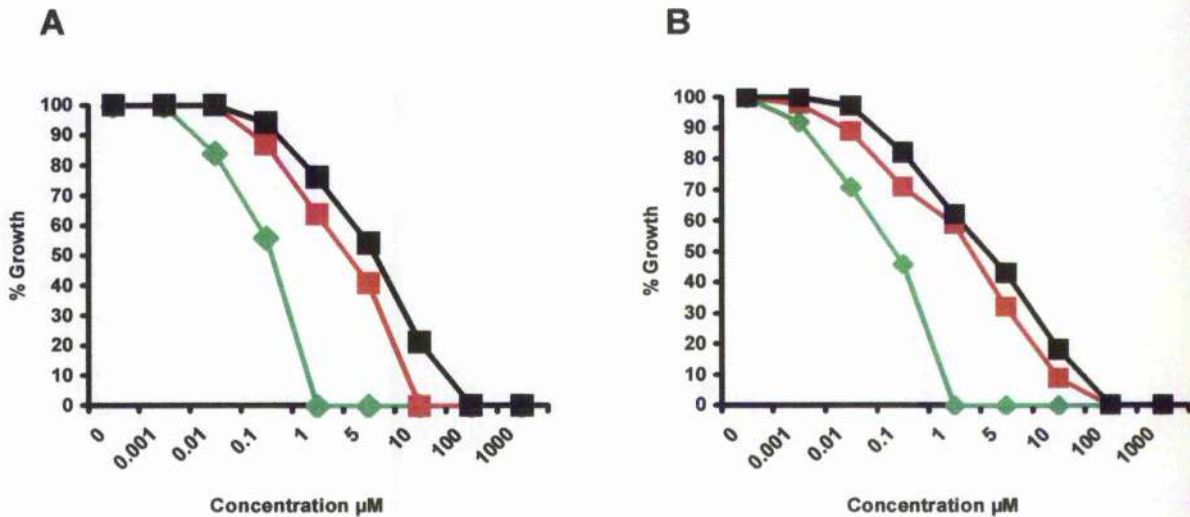
Fig 4.1 A and B: Growth Inhibition assay for the three parental lines. (A) The percentage growth inhibition in procyclic cultures with increasing doses of MelCy indicated that lines STIB 386 (■) and TREU 927 (■) expressed a resistance phenotype in comparison to line STIB 247 (◆). **(B)** The percentage growth inhibition in bloodstream cultures with MelCy giving almost identical results as those determined for the procyclic forms. The x-axis represents the percentage growth after 24hr incubation in the presence of MelCy, the y-axis represents increasing concentrations of MelCy.

Whilst the growth inhibition assay is quite reliable for determining drug susceptibility of the trypanosome lines, it is very time consuming and difficult to accurately assess the end–point of the assay. None the less, it was possible to distinguish between sensitive and resistant phenotypes in both procyclic and bloodstream forms (Fig 4.1 A and B). As over 70 progeny and seven different drugs were to be analysed so as to determine the inheritance of phenotype, it was necessary to use a more rapid assay that was both reliable and reproducible.

The results observed from the growth inhibition assay gave a good guide as to the sensitivity patterns of the three parental lines in the presence of MelCy. These would serve as an indicator as to whether the AlamarBlue assay could be used as an alternative to the growth inhibition assay. The AlamarBlue assay used here was based on a previous study (Raz *et al.*, 1997) where the determination of cell viability and proliferation in the presence of drugs was measured fluorometrically.

Using the AlamarBlue assay for screening procyclic and bloodstream forms *in vitro*, the phenotypes of the three parental lines were determined. The procyclic forms were inoculated into 96 well flat bottomed microtitre plates at a density of 2×10^6 /ml and incubated in the presence of increasing concentrations of MelCy (0.001 – 1000 µM) for 24 hr with 10% AlamarBlue dye. The conversion of the AlamarBlue dye to its fluorescent product was relatively unaffected in the range of MelCy concentrations used with the procyclic forms of lines STIB 386 and TREU 927. Thereby suggesting that there was no significant effect on cell viability or proliferation of these lines. However, for

line STIB 247 the measure of fluorescence was significantly reduced suggesting cell viability was severely affected at MelCy concentrations of 0.1 µM and above (Fig 4.2 A). Bloodstream forms were also tested *in vitro* using AlamarBlue and the pattern of susceptibility to MelCy for the three lines were the same as those determined for the procyclic forms; cell viability was relatively unaffected for STIB 386 and TREU 927 in comparison to STIB 247(Fig 4.2 B). However, with the bloodstream forms it was observed that the fluorometric reading for the three lines were slightly lower than those observed for the procyclic forms. This may have been due to the fact that these bloodstream forms have not been adapted to culture and so a reduction in viability would be expected. Given these data, it was clear that the AlamarBlue assay could be used as an alternative to the growth inhibition assay as it gave the same drug susceptibility profile for the three parental lines i.e. STIB 247 was sensitive while STIB 386 and TREU 927 were resistant to MelCy.

A

B
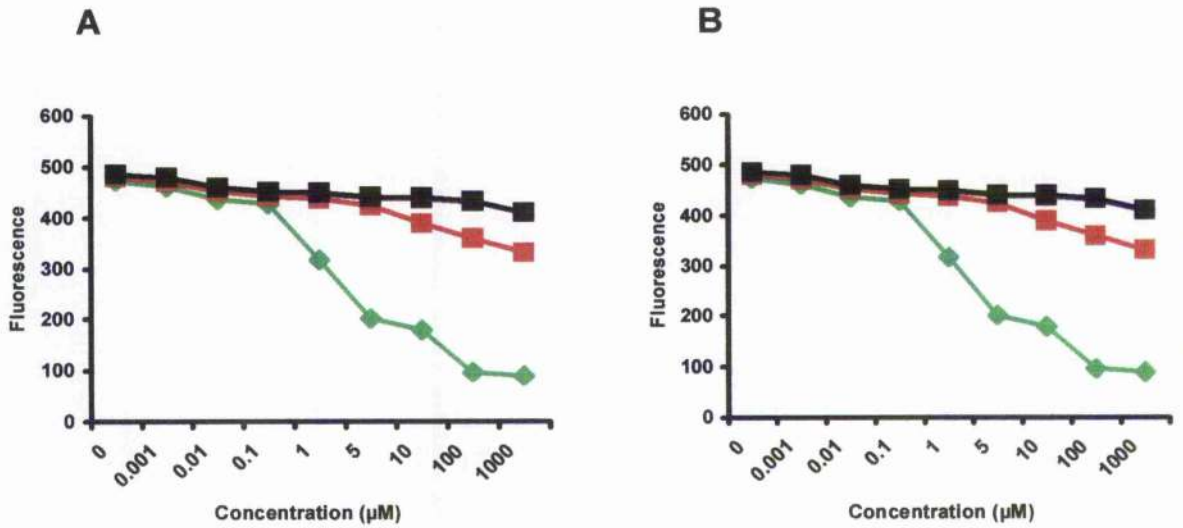
Fig 4.2 A and B: **Dose response of the three parental lines used for crosses determined using the AlamarBlue screening assay and fluorescence spectrometry.** (A) Indicates that STIB 386 (■) and TREU 927 (■) are resistant to MelCy in comparison to STIB 247 (♦). (B) The response of bloodstream forms in the presence of Cymelarsan, where STIB 386 and TREU 927 gave a resistant phenotype in comparison to STIB 247. The x-axis represents the measure of fluorescence at OD_{590}, and the y-axis represents the increasing concentrations of Cymelarsan.

To prove that the results obtained from the *in vitro* screening assays reflected those obtained, *in vivo*, assessment of drug resistance patterns for the three parental lines was carried out in mice. Groups of five immunosuppressed ICR mice were infected (i.p) with $\sim 10^5$ trypanosomes and drug treated after 24hr with MelCy ranging in concentration from 0.1 – 5 mg/kg. Following drug treatment the parasitaemia for each group of mice was monitored from tail blood for patent infection for a period of 14 days. If no trypanosomes were observed after this time period drug treatment was considered to have been effective. Drug failure was judged to have occurred if trypanosomes were observed by direct microscopy of tail blood. The results obtained were expressed as the

minimum curative dose (MIC) of MelCy required to clear the infection from all five mice in each group. The results showed that the MIC was 0.5 mg/kg for STIB 247, 5 mg/kg for STIB 386 and 5 mg/kg for TREU 927. These data indicated that STIB 386 and TREU 927 were 10 fold more resistant than STIB 247 to MelCy *in vivo* (Table 4.1). These results were consistent with those found for the *in vitro* screening assays of these three lines. On this basis, the AlamarBlue assay was used for screening of the F1 hybrid progeny to determine the inheritance of the drug resistance phenotype of the parental lines.

Stock	MelCy concentration	No. of Mice with patent infection
STIB 247	0.1 mg/kg	5/5
	0.2 mg/kg	3/5
	0.5 mg/kg	0/5
STIB 386	0.5 mg/kg	3/5
	1 mg/kg	3/5
	2 mg/kg	1/5
	5 mg/kg	0/5
TREU 927	1 mg/kg	5/5
	2 mg/kg	5/5
	5 mg/kg	0/5

Table 4.1: Screening the drug resistance phenotype of the three parental lines, *in vivo*, with increasing doses of Cymelarsan. The table indicates the results for the mouse screens (five for each concentration of MelCy) used to determine the resistance phenotype of the three parental lines. The minimum inhibitory concentration (MIC) of MelCy required to clear infection of STIB 247 was 0.5 mg/kg. The MIC of MelCy required to clear STIB 386 and TREU 927 infection was 5 mg/kg. These results confirm the findings obtained from the *in vitro* assays i.e. STIB 386 and TREU 927 display resistance phenotypes in comparison to STIB 247. A complete set of results are in Appendix Table A3.A

The *in vitro* drug response of 38 F1 hybrid progeny derived from a genetic cross between STIB 247 x TREU 927 to MelCy was determined using the AlamarBlue assay. The assay was carried out in an identical manner to that used for the testing of the three parental lines. These data showed that the progeny clearly segregated into either the resistant or sensitive phenotype (Fig 4.3). Twenty–two of the progeny followed the pattern of resistance observed for TREU 927 while 16 followed the same phenotypic pattern as the sensitive STIB 247. There were no intermediate or non–parental phenotypes observed, which suggested that one gene determined resistance (Fig 4.3). The analysis was also carried out for bloodstream forms of STIB 247, TREU 927 and 10 of the 38 F1 hybrid progeny from the cross STIB 247 x TREU 927 using the *in vitro* AlamarBlue assay. The results for each line agreed with those observed for the procyclic forms, which suggested that the mechanism determining drug resistance was the same for both bloodstream and procyclic forms (Fig 4.4).

Concentration (µM)
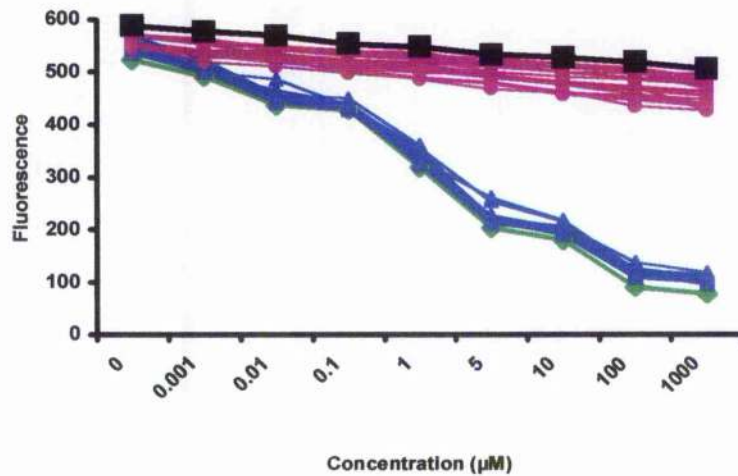
Fig 4.3: Dose response to MelCy of the hybrid progeny clones of the cross STIB 247 x TREU 927 determined by the AlamarBlue assay and fluorescence spectrometry. The results show that the progeny segregated into two phenotypes, either sensitive (▲) or resistant (•), in the same manor as either parent. There were no intermediate or non–parental phenotypes detected, which suggested that one gene governed resistance. The x-axis represents the measure of fluorescence at OD_{590}, and the y-axis represents the increasing concentrations of Cymelarsan.
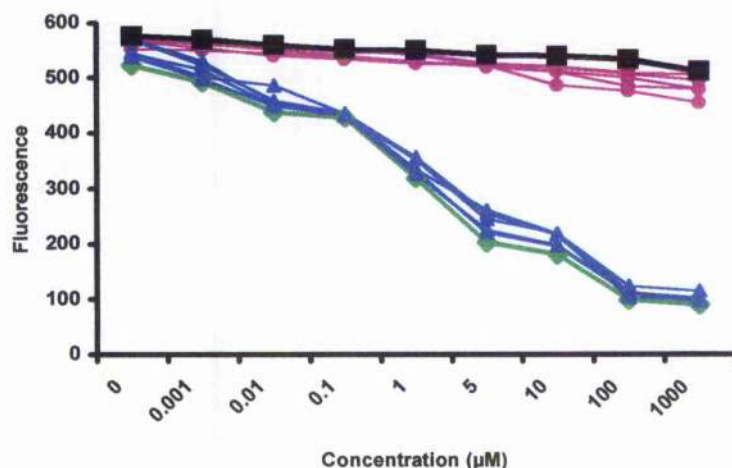
Fig 4.4: Effect of MelCy on 10 F1 hybrid progeny bloodstream forms of the STIB 247 x TREU 927 cross, *in vitro*, using the AlamarBlue screening assay. These results followed those found for the procyclic forms in that they segregated into two phenotypes and each F1 progeny clone had the same phenotype in both assays. STIB 247 (◆), TREU 927 (■), resistant F1 progeny (●) and sensitive F1 progeny (▲). The x-axis represents the measure of fluorescence at OD_{590}, and the y-axis represents the increasing concentrations of Cymelarsan.

In order to conclusively prove that the results obtained from the AlamarBlue assay for the F1 hybrid progeny reflected resistance/sensitivity, a selection of F1 hybrids were tested *in vivo*. The method used was exactly the same as that used to test the parental lines. Six F1 hybrids, three sensitive and three resistant according to the AlamarBlue results, were tested for their response to a range of MelCy concentrations (0.1, 0.2, 0.5, 1, 2 and 5 mg/kg) (Table 4.2). The three hybrids that were sensitive *in vitro* also gave a sensitive phenotype *in vivo* with infection being cleared from the five mice in this group with 0.5 mg/kg MelCy, the same as that for the parental line STIB 247. The three *in vitro* resistant progeny were also resistant *in vivo* with the infection being cleared from the group of mice at a MelCy concentration of 5 mg/kg (Table 4.2), the same as that found for the parental line TREU 927 (Table 4.1). Thus the results obtained were very clear–cut, with the hybrid progeny behaving like one or other parent, either being completely

sensitive or resistant to increasing doses of MelCy in all tests used. See Appendix A3.B

for the complete set of *in vivo* results of the hybrid progeny.

F1 Hybrid	MelCy concentration	No. of Mice with patent infection
F532/72 MCL 5 (S)	0.1 mg/kg	4/5
	0.2 mg/kg	3/5
	0.5 mg/kg	1/5
	1 mg/kg	0/5
F124/28 BCL 20 (S)	0.1 mg/kg	3/5
	0.2 mg/kg	3/5
	0.5 mg/kg	0/5
	1 mg/kg	0/5
F532/72 BCL 1 (S)	0.1 mg/kg	5/5
	0.2 mg/kg	3/5
	0.5 mg/kg	1/5
	1 mg/kg	0/5
F532/63 BCL 2 (R)	0.5 mg/kg	5/5
	1 mg/kg	4/5
	2 mg/kg	3/5
	5 mg/kg	0/5
F532/63 CL A11 (R)	0.5 mg/kg	3/5
	1 mg/kg	4/5
	2 mg/kg	2/5
	5 mg/kg	0/5
F974/78 BCL 3 (R)	0.5 mg/kg	5/5
	1 mg/kg	4/5
	2 mg/kg	2/5
	5 mg/kg	0/5

Table 4.2: Screening the drug resistance phenotype of six F1 hybrids of the STIB 247 x TREU 927 cross, *in vivo*, with increasing doses of Cymelarsan. The table indicates the results for the mouse screens (five for each concentration of MelCy) used to determine the resistance phenotype of the six F1 hybrids. The minimum inhibitory concentration (MIC) of MelCy required to clear infection of F532/72 MCL 2, F124/28 BCL 20 and F532/72 BCL 1 was 0.5 mg/kg. The MIC of MelCy required to clear F532/63 BCL 2, F532/63 CL A11 and F974/78 BCL 3 infection was found to be 5 mg/kg. These results confirm the findings obtained from the *in vitro* assays i.e. F532/72 MCL 5 and F124/28 BCL 20 displayed sensitive phenotypes and F532/63 BCL 2 and F532/63 CL A11 had resistant phenotypes. **S** – sensitive *in vitro*; **R** – resistant *in vitro*. A complete set of results are in Appendix Table A3.B

The 34 F1 hybrid progeny of the STIB 247 x STIB 386 cross were screened as procyclic forms for their resistance to MelCy using the AlamarBlue assay *in vitro* and it was found that 19 of the progeny were sensitive and 15 were resistant. The 19 sensitive progeny expressed MelCy sensitivity with levels equivalent to those found for the parental line STIB 247. The 15 resistant progeny expressed the same phenotypic pattern as the resistant parental line STIB 386 (Fig 4.5). Once again there were no intermediate or non –parental phenotypes detected. Ten of the F1 hybrid progeny were also tested *in vitro* using bloodstream forms. The phenotypes obtained for these hybrids were the same as those found for the equivalent procyclic forms of the same hybrids (Fig 4.6).

Concentration (µM)
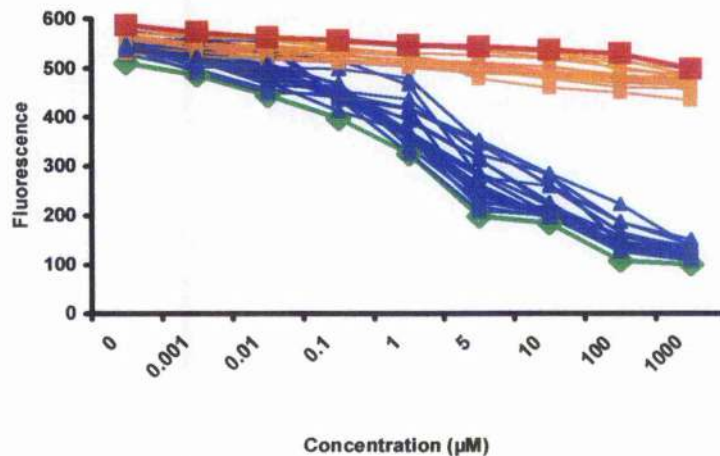
Fig 4.5: Dose response to MelCy of the hybrid progeny clones of the cross STIB 247 x STIB 386 determined by the AlamarBlue assay and fluorescence spectrometry. The results show that the progeny segregated into two phenotypes, either sensitive or resistant, in the same manor as either parent. There were no intermediate or non–parental phenotypes detected, which suggested that one gene governed resistance. STIB 247 (◆), STIB 386 (■), resistant F1 progeny (■) and sensitive F1 progeny (▲). The x-axis represents the measure of fluorescence at OD_{590}, and the y-axis represents the increasing concentrations of Cymelarsan.
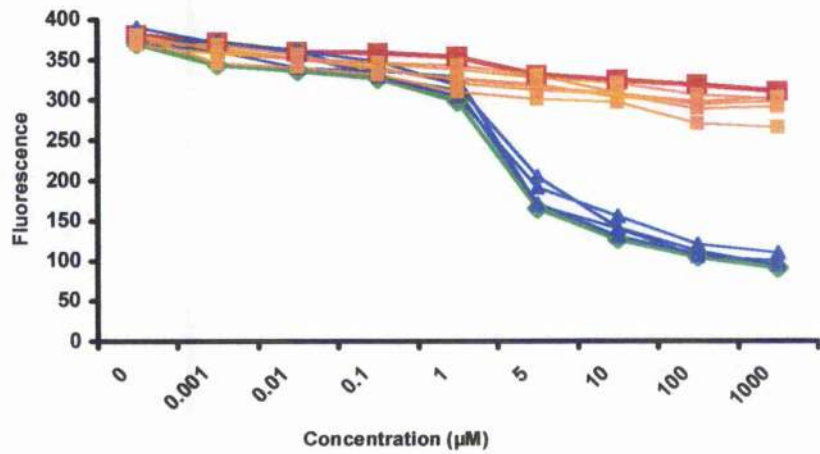
Fig 4.6. Effect of MelCy on 10 F1 hybrid progeny bloodstream forms of the STIB 247 x STIB 386 cross, *in vitro*, using the AlamarBlue screening assay. These results followed those found for the procyclic forms in that they segregated into two phenotypes and therefore backed up the results found using the AlamarBlue assay. STIB 247 (♦), TREU 927 (■), resistant F1 progeny (■) and sensitive F1 progeny (▲). The x-axis represents the measure of fluorescence at OD_{590}, and the y-axis represents the increasing concentrations of Cymelarsan.

As with the hybrids from the STIB 247 x TREU 927 cross, six hybrid progeny (three sensitive and three resistant), were tested *in vivo* for their response to a range of concentrations of MelCy (0.1, 0.2, 0.5, 1, 2 and 5 mg/kg). The results obtained agreed with those found from the *in vitro* assays. The three sensitive hybrids also expressed a sensitive phenotype *in vivo* with infection being cleared from the group of five mice at a concentration of 0.5 mg/kg MelCy, the same as that found for the parental line STIB 247. The three *in vitro* resistant hybrids also expressed a resistant phenotype *in vivo* with infection being cleared at a concentration of 5 mg/kg MelCy the same as that previously found for the parental line STIB 386 (Table 4.3).

103

F1 Hybrid	MelCy concentration	No. of Mice with patent infection
F9/45 MCL 11 (S)	0.2 mg/kg	2/5
	0.5 mg/kg	1/5
	1 mg/kg	0/5
F492/50 BCL 23 (S)	0.2 mg/kg	3/5
	0.5 mg/kg	0/5
	1 mg/kg	0/5
F19/31 BCL 11 (S)	0.2 mg/kg	3/5
	0.5 mg/kg	1/5
	1 mg/kg	0/5
F492/50 BCL 8 (R)	0.5 mg/kg	5/5
	1 mg/kg	4/5
	2 mg/kg	4/5
	5 mg/kg	1/5
F492/50 BCL 21 (R)	0.5 mg/kg	5/5
	1 mg/kg	4/5
	2 mg/kg	1/5
	5 mg/kg	0/5
F9/41 BCL 7 (R)	0.5 mg/kg	5/5
	1 mg/kg	3/5
	2 mg/kg	2/5
	5 mg/kg	0/5

Table 4.3: Screening the drug resistance phenotype of six F1 hybrids of the STIB 247x TREU927 cross, *in vivo*, with increasing doses of Cymelarsan. The table indicates the results for the mouse screens (five for each concentration of MelCy) used to determine the resistance phenotype of the six F1 hybrids. The minimum inhibitory concentration (MIC) of MelCy required to clear infection of F9/45 MCL 11, F492/92 BCL 23 and F19/31 BCL 11 was 0.5 mg/kg. The MIC of MelCy required to clear F492/50 BCL 8, F492/50 BCL 21 and F9/41 BCL 7 infection was found to be 5 mg/kg. These results confirm the findings obtained from the *in vitro* assays i.e. F9/45 MCL 11 and F492/50 BCL 23 displayed sensitive phenotypes and F492/50 BCL 8 and F492/50 BCL 21 had resistant phenotypes. S – sensitive *in vitro*; R – resistant *in vitro*. A complete set of results are in Appendix Table A3.C

As cross resistance both between different arsenicals, Berenil and Pentamidine has been widely reported in the literature, experiments were undertaken to determine if there was any cross–resistance between MelCy and other trypanocides namely, melarsoprol (MelB), trimelarsan (MelW), melarsen oxide (MelOx), pentamidine, diminazene aceturate (Berenil) and phenylarsenoxide (PhenOx). Analysis was initially carried out on the parental lines STIB 247 and TREU 927. As can be observed from Fig 4.7 A – E there was a rapid decrease in cell viability for line STIB 247 at concentrations of 1 µM and above for MelB, MelW, MelOx, Pentamidine and Berenil. With PhenOx, however, cell viability decreased at concentrations of 0.001 µM and above for STIB 247, suggesting that PhenOx is much more potent that the melaminophenyl arsenicals (Fig 4.7 F). In comparison the results obtained for the MelCy resistant line TREU 927 indicated that MelW, Pentamidine and Berenil had no marked effect on cell viability at concentrations up to 100 µM, the same as those observed for MelCy, while an effect with PhenOx was only observed at 1 µM (Fig 4.7 B, D, E and F). With MelB and MelOx, however, cell viability began to decrease at concentrations of 1 µM but never reached the same levels of reduced viability as those observed for STIB 247 (Fig 4.7 A and C). The drug screening assay of the hybrid progeny of the STIB 247 x TREU 927 cross revealed that the resistant phenotype correlated with either parent with the same progeny being resistant to MelB, MelW, MelOx, Berenil and PhenOx as MelCy. Similarly those that were sensitive to MelCy were also sensitive to MelB, MelW, MelOx, Berenil and PhenOx (Fig 4.7 A – C, E and F). As with MelCy there were no intermediate or non–parental phenotypes detected. The results for Pentamidine showed that all the hybrid progeny had a sensitive phenotype following the same pattern as that found for

STIB 247 (Fig 4.7 D). This result shows that sensitivity is dominant to resistance in the progeny, implying that a different locus determines the resistance phenotype for this diamidine.

Fig 4.7 A – F: Determination of cross-resistance between MelCy and six other trypanocidal drugs for the F1 hybrid progeny clones of the STIB 247 (◆) x TREU 927 (■) cross; melarsoprol (A), trimelarsan (B), melarsen oxide (C), pentamidine (D), berenil (E) and phenyl arsenoxide (F), using the AlamarBlue screening assay.
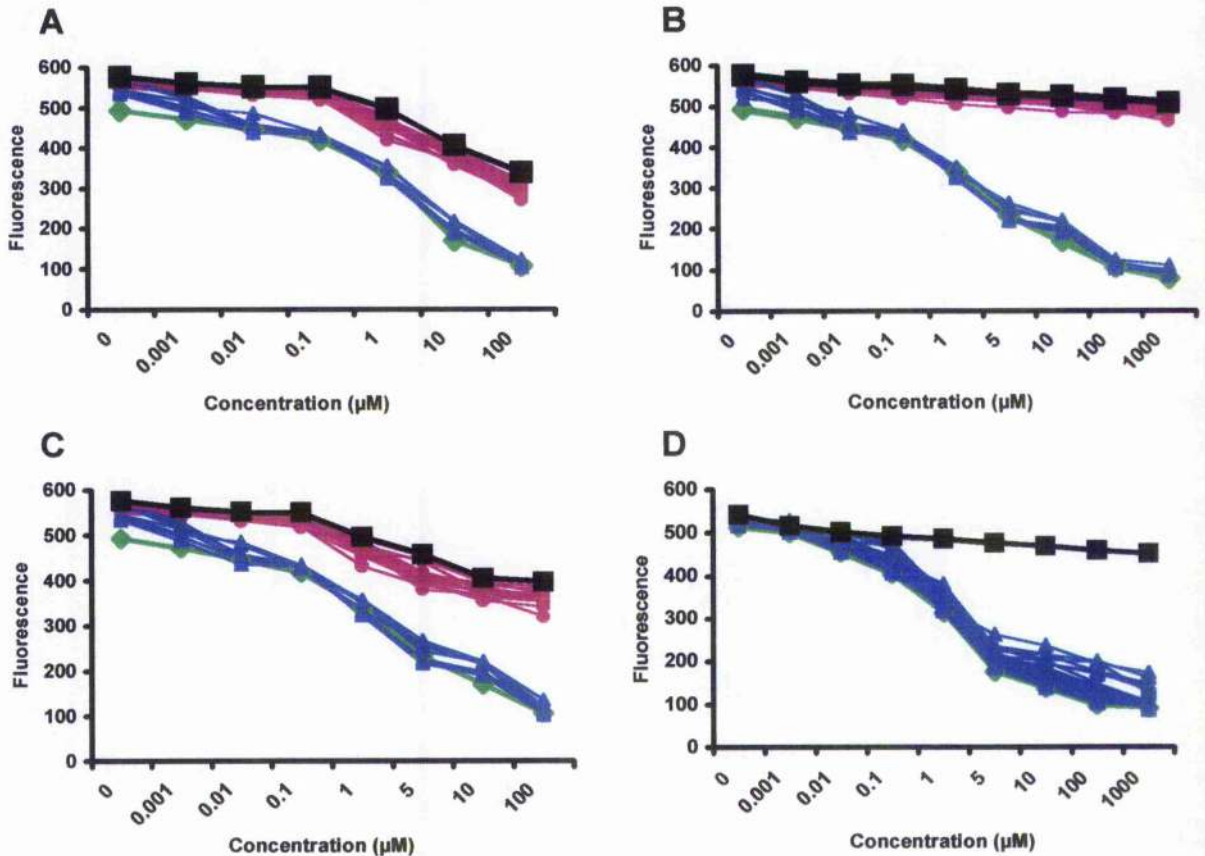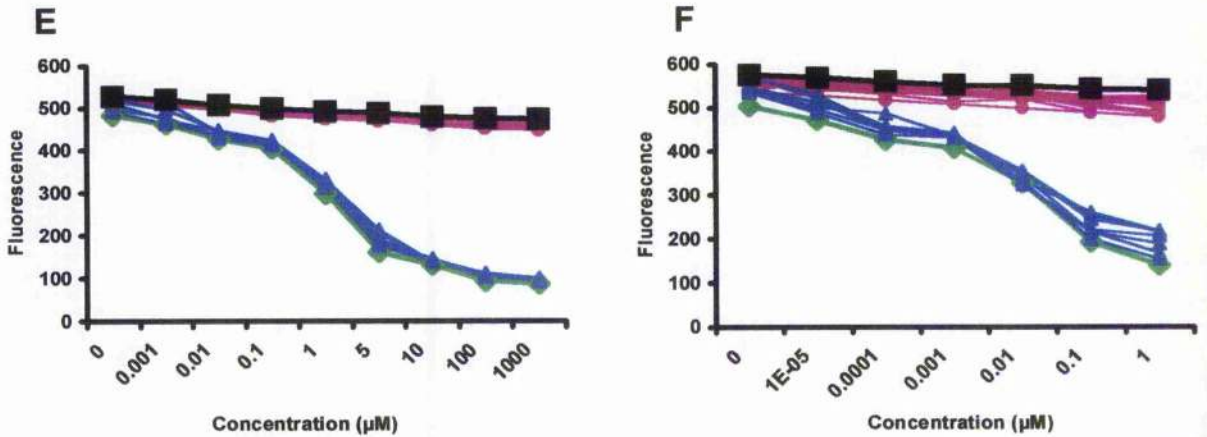
Fig 4.7 A-F: These graphs indicated that there was cross-resistance to all but one of the drugs tested, as the progeny segregated in a 1:1 ratio the same as that found for MelCy. The exception to this finding was pentamidine where all the progeny were sensitive (**D**). STIB 247 (◆), TREU 927 (■), resistant F1 progeny (●) and sensitive F1 progeny (▲). The x-axis represents the measure of fluorescence at OD_{590}, and the y-axis represents the increasing concentrations of each drug.

An identical analysis was carried out for STIB 386 and the hybrid progeny derived from the STIB 247 x STIB 386 cross. Firstly, for STIB 386, incubation with MelW, Pentamidine and Berenil showed no marked effect on cell viability at concentrations of 100 µM and above (Fig 4.8 B, D and E). For MelB and MelOx cell viability began to decrease at concentrations of 1 µM and above but as with TREU 927 never reached the same levels of sensitivity as STIB 247 (Fig 4.8 A and C). With PhenOx, STIB 386 cell viability began to decrease at concentrations of 0.001 µM and above but this stock was still 100 fold more resistant than STIB 247 (Fig 4.8 F). Therefore, STIB 386 was more sensitive to PhenOx than TREU 927 (Figs 4.7 and 4.8 F). Analysis of the progeny from this cross, indicated that those progeny which were resistant to MelCy were also resistant to the six other drugs tested. Similarly those that were sensitive to MelCy were also sensitive to the other six drugs. The one difference between the progeny of the two

crosses was that the inheritance of Pentamidine resistance was different in the STIB 247 x TREU 927 cross compared to the STIB 247 x STIB 386 cross. In the latter cross co– segregation of Pentamidine resistance with the resistance to the other six drugs was observed.

Fig 4.8 A – F: Determination of cross-resistance between MelCy and six other trypanocidal drugs for the F1 hybrid progeny clones of the STIB 247 (■) x STIB 386 (■) cross; melarsoprol (A), trimelarsan (B), melarsen oxide (C), pentamidine (D), berenil (E) and phenyl arsenoxide (F), using the AlamarBlue screening assay.

E

F
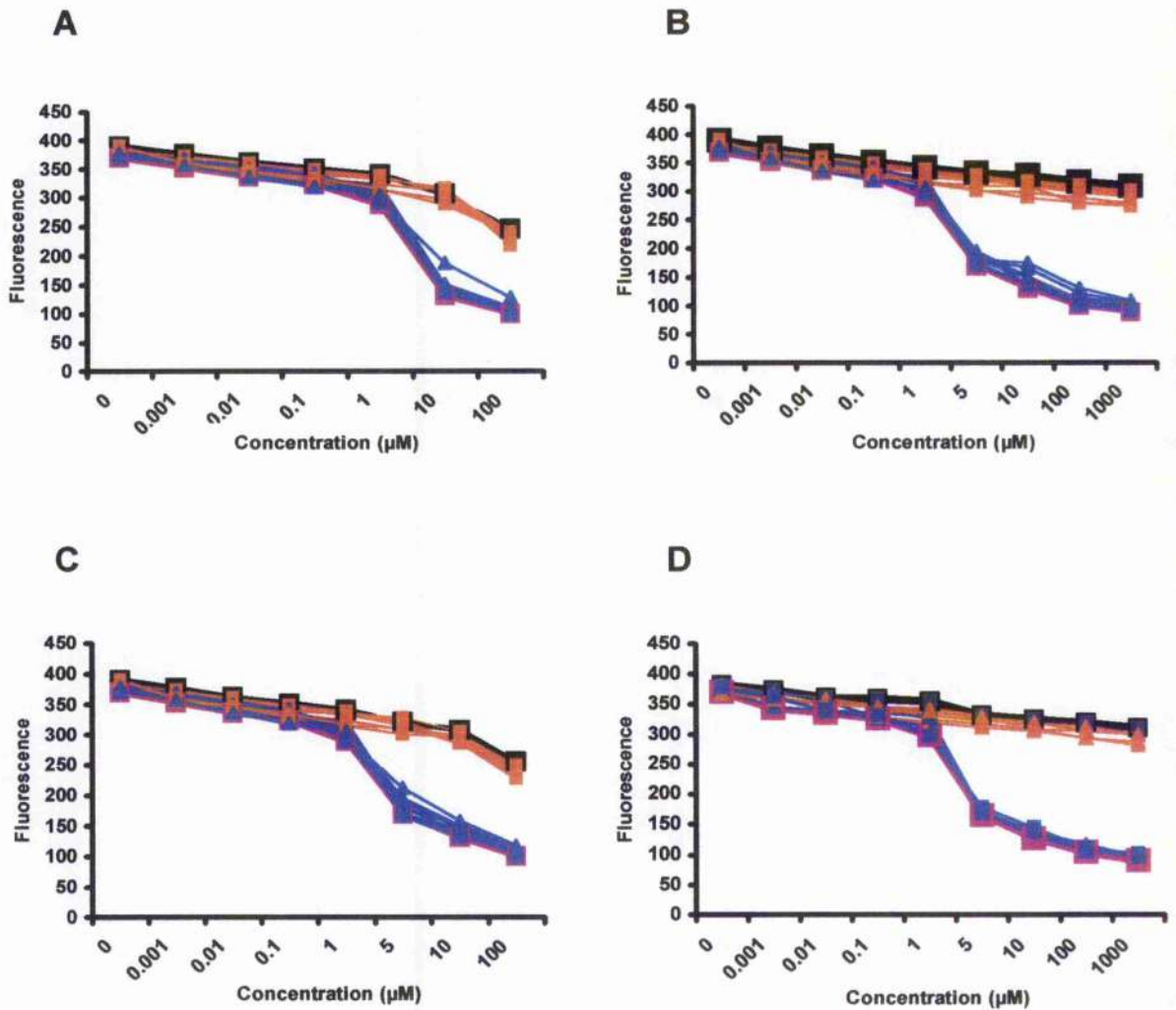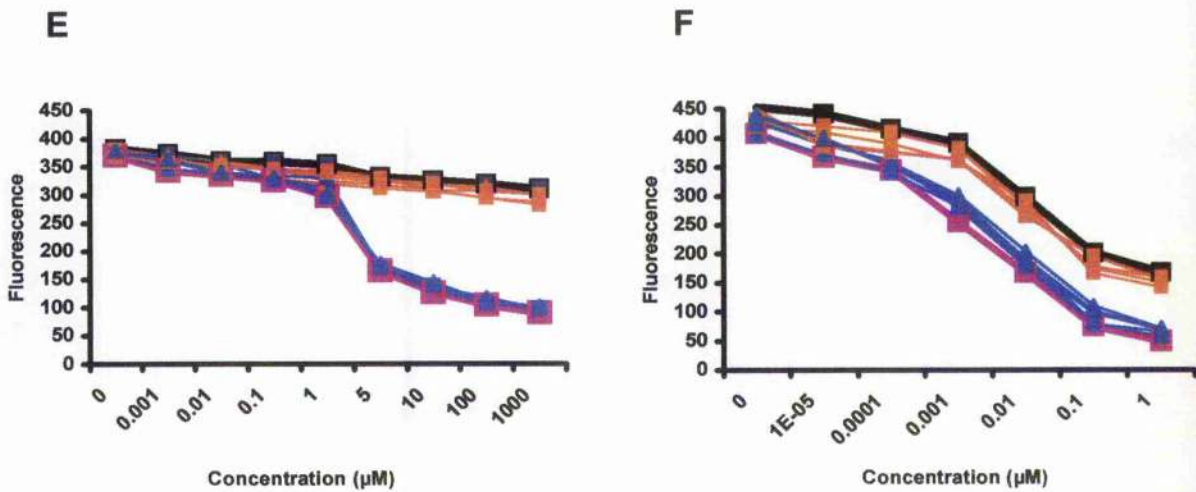
Fig 4.8 A-F: These graphs indicated that there was cross-resistance to all the drugs tested as the progeny segregated in a 1:1 ratio the same as that found for MelCy. The interesting result found was that for this cross the progeny segregated in a 1:1 ratio for pentamidine (**D**), which was not the case for the STIB 247 X TREU 927 cross. Therefore, suggesting that resistance to pentamidine was governed by more than one gene. STIB 386 (■), STIB 247 (■), Resistant F1 progeny (■) and Sensitive F1 progeny (▲). The x-axis represents the measure of fluorescence at OD_{590}, and the y-axis represents the increasing concentrations of each drug.

With these sets of data, a genetic model of inheritance for MelCy resistance could be proposed. The pattern of the segregation of phenotype in the progeny allows models for the number of loci and alleles involved in conferring the trait of interest to be defined. As the progeny segregate into only the two parental phenotypic classes, a single locus model can be put foreward as any multilocus model is likely to generate progeny with levels of resistance intermediate between those of the parental lines.

The simplest model for the inheritance of the drug resistance phenotype in the F1 progeny that fits the experimental data would be where the resistance phenotype is determined by two alleles at a single locus with the resistant parent being heterozygous

for a dominant resistant allele and the sensitive parent being homozygous for a sensitive allele, as illustrated in Fig 4.9.

Based on this model, half the F1 progeny would inherit the dominant resistance allele and half would inherit the recessive sensitive allele leading to a prediction of a 1:1 segregation of resistant:sensitive phenotypes in the progeny. The observed segregation for the cross between STIB 247 x TREU 927 is 16 sensitive progeny and 22 resistant progeny and this does not differ significantly from a 1:1 ratio as determined by χ^2. Thus, the data obtained support a single locus model in which the resistant TREU 927 parental line is heterozygous for a dominant allele determining resistance to the four arsenical compounds and Berenil but not Pentamidine (Fig 4.7 and Fig 4.9). Analysis of the progeny of the cross between STIB 247 and STIB 386 found that there were 19 sensitive progeny and 15 resistant progeny. Therefore, the same conclusions can be drawn from this cross i.e. a single locus model in which the resistant STIB 386 parental line is heterozygous for a dominant allele determining resistance to the four arsenicals, Berenil and Pentamidine (Fig 4.8 and Fig 4.9).

```
Parent - Phenotype        S(247)  x  R(927)

     Genotype               ss    x    Rs

F1 phenotype                 S    x    R
Genotype                 ss  ss   x  sR sR
Predicted ratio              1    :    1
Observed ratio              16    :    22
```

Figure 4.9 : A predicted Genetic Model for Cymelarsan Resistance. A genetic model for Cymelarsan resistance for 247 x 927 was determined. One parent must be heterozygous otherwise the data set would be either all sensitive or all resistant. The progeny are inherited in 1:1 ratio (chi^2 = 0.52, df =1, p>0.05).

The availability of progeny from a cross between the two resistant lines (STIB 386 and TREU 927) allows the genetic models for the inheritance in the two crosses between each of these resistant lines and the sensitive line to be tested without the need for a backcross or F2 progeny. As only seven independent progeny were available from the STIB 386 x TREU 927 cross, it is difficult to analyse the segregation statistically. However, two sensitive progeny were obtained from the cross between the two resistant parents, providing strong evidence, based on a single locus model, that the two parental lines are heterozygous with a sensitive recessive allele (Fig 4.10). If the models are correct, a cross between two resistant lines that are heterozygous for a dominant resistant allele should yield progeny that segregate 1:3 between sensitive and resistant phenotypes (Fig 4.11). These data, therefore, validate the genetic model proposed for both parental resistant lines.
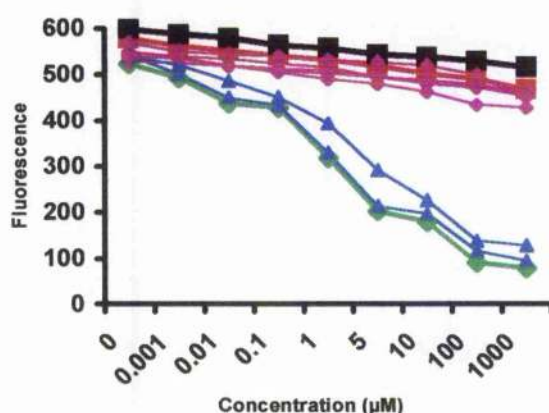
Fig 4.10: Dose response to MelCy of the seven hybrid progeny clones from the cross STIB 386 x TREU 927 determined by the AlamarBlue assay. The results indicate that two of the progeny (▲), followed the sensitive phenotype found for the STB 247 parental line (◆), whilst five progeny (◆) followed the phenotype found for the two resistant parental lines STIB 386 (■) and TREU 927 (■). These data confirm the proposed genetic model for both parental resistant phenotypes. The x-axis represents the measure of fluorescence at OD_{590}, and the y-axis represents the increasing concentrations of Cymelarsan.

Parental - Phenotype   R(386)   x   R(927)

Genotype   sR   sR

F1 phenotype	S	R
Predicted Genotype ratio	ss x	sR sR RR
Predicted phenotype ratio	1 :	3
Observed phenotype ratio	2 :	5

Fig 4.11: Genetic cross of the two resistant lines to validate the predicted genetic model of inheritance of drug resistance phenotype. If the genetic model was correct, where resistance was heterozygous and dominant then a cross between the two resistant lines would be expected to produce some sensitive progeny. As can be seen two of the progeny derived from the cross between STIB 386 x TREU 927 were sensitive to MelCy, thereby validating the genetic model of inheritance (Fig 4.9).

This model, however, did not fit with the data obtained for the pentamidine phenotype of the progeny for the STIB 247 x TREU 927 cross where all the progeny were sensitive (Fig 4.3 D). These data suggested that another locus was involved in conferring pentamidine resistance situated on another region of the genome, however it was not possible to identify this locus due to its lack of segregation. There are two single locus models of inheritance that would fit this data that are detailed in Fig 4.12. However, these models could only be tested by generating F2 or backcross progeny. It is also formally possible that the same locus as that determining MelCy resistance in TREU 927 determines Pentamidine resistance but that the dominance relationships in the allele in TREU 927 is different from that in STIB 386.

STIB 247   x   TREU 927
SS   x   rr
SS   x   rS

Figure 4.12 : Models for Pentamidine. The only genetic models for Pentamidine which can be used for S being dominant. These indicate that for S to be dominant it must be homozygous.

4.4 DISCUSSION

Initially the *in vitro* growth inhibition assay was used to determine the phenotype of the three parental lines, STIB 247, STIB 386 and TREU 927, for both procyclic and bloodstream forms. It was found that STIB 386 and TREU 927 were resistant to MelCy in comparison to line STIB 247 in both life cycle stages (Fig 4.1). Whilst this screening assay was reliable and reproducible, it was very time consuming and with the numbers of hybrid progeny requiring testing with MelCy and five other drugs, a more rapid screening assay was required.

The AlamarBlue assay had previously been used to determine drug sensitivity of trypanosomes (Raz *et al.*, 1997) and found to be very effective. It was, therefore, decided to test this assay to see if the results obtained for the three parental lines were consistent with those determined from the growth inhibition assay. The AlamarBlue assay is essentially a measure of cell viability based on the reduction of the dye to a fluorescent form by viable cells with the measurement of this product fluorometrically. Therefore, if the cell viability was compromised by the presence of increasing doses of drug then the dye would not be reduced to its fluorescent form. As can be seen from the results, the same phenotypes were observed using the AlamarBlue assay as those found from the growth inhibition assay i.e. STIB 386 and TREU 927 were resistant to MelCy in comparison to STIB 247 for both procyclic and bloodstream forms (Fig 4.2). The AlamarBlue screening assay was, therefore, found to be reliable, reproducible and much less time consuming than the growth inhibition assay.

The differences between the three parental lines allowed the inheritance of the drug resistant phenotype to be determined by the analysis of the hybrid progeny generated from genetic crosses between the three lines could be determined. Analysis of the 38 F1 hybrid progeny generated from the STIB 247 x TREU 927 cross (Chapter 2), showed that 22 of the progeny followed the same resistance pattern as that found for the parental line TREU 927 while the remaining 16 progeny were sensitive and of the same phenotype as STIB 247 (Fig 4.3). There were no intermediate or non-parental phenotypes detected, suggesting that a single locus conferred drug resistance. The same pattern of inheritance was found for bloodstream forms *in vitro*, using the AlamarBlue assay.

The results obtained from the *in vitro* screening of phenotype were backed up *in vivo*. The parental lines and three sensitive and three resistant progeny were tested for their response to increasing doses on MelCy, *in vivo*. As can be seen from Table 4.2 the minimum curative dose of MelCy required to clear infection with STIB 247 and the three sensitive progeny was 0.5 mg/kg, whilst a concentration of 5 mg/kg was required to clear infection for TREU 927 and the three resistant progeny. These results therefore suggested that the same mechanism is involved in conferring the drug resistance phenotype in both procyclic and bloodstream forms using either *in vivo* or *in vitro* assays.

Using the same screening assays the parental line STIB 386 and the 34 F1 hybrid progeny derived from the genetic cross, STIB 247 x STIB 386 were also analysed. It

was shown that 19 of the progeny gave a sensitive phenotype, following the same levels as that found for the parental line STIB 247, while the remaining 15 progeny had a similar resistance to the parental line STIB 386 (Fig 4.5). Once again there were no intermediate or non-parental phenotypes detected. Six hybrid progeny (three sensitive and three resistant) and the parent STIB 386 were also tested *in vivo* for their response to increasing doses of MelCy. The results were consistent with those found using the *in vitro* screening assay i.e. STIB 386 and the three resistant progeny were resistant by comparison to STIB 247 and the three sensitive progeny (Table 4.3 and Appendix Table A3.C).

The results obtained from the screening of the hybrid progeny derived from the two genetic crosses revealed several interesting findings. Firstly, the segregation of resistant or sensitive phenotypes in the progeny indicated that a single locus was involved in conferring the drug resistance trait. Secondly, the drug resistance trait was inherited as 15 and 22 of the progeny followed the same phenotypic pattern of resistance as the parental lines STIB 386 and TREU 927, respectively. Finally, previous studies have indicated that the P2 transporter is involved in conferring drug resistance (Carter et al., 1999; de Koning et al., 2000) but P2 activity is not detected in procyclic forms (de Koning, 2001). However, the results obtained in this study clearly indicated that procyclics as well as bloodstream forms express a resistant phenotype, thus suggesting that there are other mechanisms by which the drug resistance phenotype arises in trypanosomes.

These data showed that the level of resistance to MelCy was consistently lower in STIB 247 than STIB 386 and TREU 927. There are a number of possible explanations for the differences in phenotype between the lines. STIB 386 was isolated from a human in West Africa and is a Type 2 *T. b. gambiense* (Gibson, 1986; Hide, 1990) and TREU 927 was isolated from a tsetse fly and found to be of intermediate resistance to human serum (VanHamme *et al.*, 2003; Turner *et al.*, In Press). Due to the fact that these are both human infective forms of *Trypanosoma brucei*, it could be assumed that they had been exposed to melarsoprol (structurally related to MelCy) treatment. TREU 927 could also have been exposed to Berenil if it had previously infected cattle or to arsenicals if it had infected a human. As STIB 247 was isolated from a hartebeest and game are not treated, it would not have been likely to have been previously exposed to drug selection therefore the lower level of resistance expressed by STIB 247 would be expected.

The next stage of this analysis was to determine if there was any cross–resistance between the arsenicals and the diamidines, Berenil and Pentamidine. It should be noted that these comparisons were carried out *in vitro* only. As can be observed from the results (Fig 4.7 A-F) there was cross–resistance and co–segregation of phenotype for MelCy, MelB, MelW, MelOx, PhenOx and Berenil for the STIB 247 x TREU 927 cross. One noticeable difference in the resistance profiles of these drugs was that the concentration of PhenOx required to bring about a loss in cell viability was much lower (1μM) for the resistant lines. PhenOx is a lipid–soluble arsenical, which lacks a melaminyl–group and therefore can cross the trypanosome membrane barrier by diffusion causing a loss in cell viability at much lower concentrations than the other

diffusion causing a loss in cell viability at much lower concentrations than the other melaminophenyl arsenicals and diamidines (Fairlamb et al., 1992). The trypanosome membrane acts as a permeability barrier and selective drug uptake mechanisms exist. However, this does not suggest that only one route exists for the uptake of the melaminophenyl arsenicals. If this were the case then it would be expected that all the drugs would have had the same levels of resistance, but MelCy was more potent than MelOx, this could be because they have better action on the intracellular target, varying diffusion rates into the cell and different affinities for the transporter given they have different structures (Fig 1.2) and EC_{50} values. The suggestion that different routes/mechanisms are involved in conferring the resistance phenotype follows the proposal that the P2 transporter is not the only route by which resistance comes about.

Another very interesting finding arising from the analysis of cross–resistance for the STIB 247 x TREU 927 cross was that all 38 hybrid progeny were sensitive to Pentamidine but the parental line TREU 927 remained resistant (Fig 4.6 D). Studies carried out on the T. brucei RU15 clone, which does not express P2 activity, showed that this line was resistant to melaminophenyl arsenicals but not to Pentamidine (Fairlamb et al., 1992). It was also found that field and laboratory strains that were resistant to melaminophenyl arsenicals were usually cross–resistant to Berenil but not to Pentamidine (Bacchi and Yarlett, 1993). These data led to the hypothesis that Pentamidine must be taken up by a route other than the P2 transporter (de Koning, 2001), which would further support the findings of this study. This hypothesis, however, could not explain why the TREU 927 parental line expressed resistance to Pentamidine

whilst the hybrid progeny were all sensitive. An explanation for this result could be that a different gene may be involved in conferring Pentamidine resistance in TREU 927. Alternatively an explanation could be that the sensitive alleles in STIB 247 are dominant over the resistant alleles in TREU 927 in relation to Pentamidine resistance/sensitivity.

Analysis of the 34 hybrid progeny derived from the STIB 247 x STIB 386 cross for cross–resistance patterns indicated that the phenotype segregated in the same manner for all the drugs tested including pentamidine i.e. 19 sensitive and 15 resistant. The only difference was that STIB 386 and the resistant progeny were more sensitive to PhenOx than TREU 927 and the resistant progeny of the STIB 247 x TREU 927 cross (Figs 4.7 F and 4.8 F). For the most part, however cross–resistance does exist between the melaminophenyl arsenicals and diamidines.

Finally, with the inheritance of phenotype data a model for the genetic basis for resistance could be proposed. As can be seen from the data, the progeny segregated in a 1:1 ratio (sensitive:resistant) as would be expected in a diploid Mendelian system. Using the data, a simple single locus genetic model was constructed (Fig 4.9), in which STIB 386 and TREU 927 are heterozygous for a dominant resistance allele. In order to prove this model was correct and in the absence of a backcross, progeny from a cross between the two resistant lines, STIB 386 and TREU 927 were analysed (Rs x Rs). Seven progeny were available from this cross and it would be expected at least some of these progeny would have a sensitive phenotype if the proposed model was correct. After screening of these progeny it was found that two had a sensitive phenotype and

five had a resistant phenotype (Fig 4.10), which is consistent with the model and establishes that both resistant stocks must have a sensitive allele. (This provides direct evidence that each parental line had a sensitive allele at the locus). Two models have been proposed for the data obtained for the results with Pentamidine for the STIB 247 x TREU 927 cross (Fig 4.12), but as previously stated either F2 or backcross progeny would need to be generated in order to determine which model is correct.

In conclusion, with these data the next step for the determination of the genetic basis of drug resistance was to map this locus initially to a chromosome and then a region within the chromosome of the *T. brucei* genome by linkage analysis followed by positional cloning. This analysis is the subject of the following chapters.

CHAPTER 5

POSITIONAL CLONING OF A LOCUS FOR RESISTANCE TO CYMELARSAN

5.1 INTRODUCTION

Positional cloning of the drug resistant trait involves identifying loci at which alleles co-segregate with the drug resistance trait in the progeny of crosses. Logically, this involves three steps: scanning the genome, chromosome by chromosome, using polymorphic genetic markers to construct a genetic map; analysis of the linkage between the drug resistance phenotype and each marker interval on the genome and finally identification of the loci which shows linkage. Linkage can be expressed in a number of ways, but the traditional statistic is the log odds of difference (LOD) score. It is generally accepted that a LOD score of more than three is considered as significant linkage (Lander and Kruglyak, 1995).

The availability of partial genetic maps for each of the chromosomes, using mini and microsatellite markers (Hall *et al.*, 2003; El-Sayed *et al.*, 2003; MacLeod and Tweedie, unpublished results) and significant numbers of recombinant progeny (Chapter 2) allow linkage analysis to be undertaken. All the progeny had been previously phenotyped for resistance to Cymelarsan and shown to segregate in a 1:1 ratio. The model for the inheritance of resistance predicted that the resistance allele was dominant at a single heterozygous locus in each of the strains STIB 386 and TREU 927 (Chapter 4). Therefore, the linkage between the phenotype of the progeny and the molecular markers on the genetic maps could be analysed, which would allow the identification of

the region of the genome that determines the arsenical resistance phenotype in *T. brucei*. The statistical significance of such linkage can be determined from the LOD score and the region of the genome containing the gene located from the position of the linked markers. The availability of the genome sequence meant that the physical location of linked markers could be defined on chromosomes and from this the open reading frames (ORFs) that lay between the flanking markers could be identified.

Mapping trait loci by genetic analysis is dependent upon the segregation relationships between polymorphic markers and the phenotype under investigation in the progeny of laboratory generated crosses of phenotypically distinct parental lines. Factors that determine the ease with which trait conferring loci can be defined include the number of loci involved and the availability of sufficient numbers of progeny to make any result statistically significant. For example, if a trait is heritable and a single locus determines the majority of phenotypic variation, then polymorphic markers, linked to a genomic segment carrying that gene, will co-segregate with the phenotype. This will pinpoint a crossover defined interval that carries the gene determining the trait. This situation has been identified in mapping chloroquine resistance in *Plasmodium falciparum* where it was shown that a single locus determined the resistance phenotype (Ferdig and Su, 2000). The determinant involved in chloroquine resistance was initially mapped to an interval of 400 kb on chromosome VII using 16 progeny from a cross and 88 restriction fragment length polymorphic (RFLP) markers (Wellems *et al.*, 1991). Further development of microsatellite markers and isolation of more than 1000 additional progeny identified five meiotic crossover events at various points along the 400 kb

segment. Comparisons of chloroquine responses in five of the progeny with marker inheritance patterns identified a locus controlling drug response that lay between two markers. These markers defined a 36 kb segment of chromosome VII and identified open reading frames (ORFs) of eight potential resistance genes (Su *et al.*, 1997).

A similar study has been conducted in *Toxoplasma gondii* (*T. gondii*) where analysis of 26 independent recombinant progeny, from two crosses between an acute virulent strain (type I) and a chronic virulent strain (type III), were analysed using 112 polymorphic markers across the 11 chromosomes of *T. gondii* to elucidate the genetic basis of acute virulence. Acute virulence, defined by cumulative mortality, was linked to loci on chromosome VII by association with marker inheritance. The locus responsible for virulence was located between two markers on chromosome VII, which were 14 map units apart (equivalent to 1.5 Mb) and accounted for approximately 50% of the variance seen in virulence between the progeny. In addition, when considered as a strict Mendelian trait (i.e. $\pm$ scoring), the phenotype of uniform lethality had a LOD score of 3.9 to one of the markers, which is considered highly significant linkage to the virulence trait (Su *et al.*, 2002). Genetic linkage analysis of a simple Mendelian trait has also been examined in *Eimeria tenella* for the inheritance of 443 polymorphic markers in 22 recombinant cloned progeny to determine the gene/genes responsible for both the rapid completion of the life cycle (precocious development) and resistance to aprinocid. In avian *Eimeria* spp, intracellular growth is shortened by serial selection for the first parasites to complete the transition from asexual to sexual reproduction (yielding precocious lines) leading to lines that enter the sexual cycle significantly faster than

those of the parents. Preliminary analysis suggests that precocious development is linked to chromosome II and drug resistance to chromosome I (Shirley and Harvey, 2000).

Partial genetic maps for TREU 927 were already available for chromosomes I, II, III, IV, IX and X with 12, 5, 16, 19, 17 and 12 informative polymorphic markers on each chromosome, respectively (MacLeod and Tweddie, unpublished results). The plan was, therefore, to determine if there was any linkage of the Cymelarsan resistance phenotype, initially to any of these chromosomes and then to a marker defined interval on the chromosome. Fine scale mapping between the markers of interest should narrow the marker defined region further thus pinpointing a crossover defined interval carrying the gene determining the Cymelarsan resistance trait.

5.2 MATERIALS AND METHODS

5.2.1 Linkage analysis

Linkage was assessed between phenotype (resistance to Cymelarsan) and genotype for all the F1 hybrid progeny derived from the genetic crosses STIB 247x STIB 386 and STIB 247 x TREU 927. As the drug resistance trait is heritable and a single locus seems to control most of the phenotypic variation in the progeny, then the polymorphic markers linked to a segment in the genome will co–segregate with the phenotype, thus pinpointing a crossover defined interval that carries the gene/genes controlling the inheritance of the trait. The significance of linkage analysis was calculated in order to obtain a LOD score value. The formula for calculating the LOD score is given below.

5.2.2 LOD score

LOD scores are used to determine if there is significant linkage for the trait of interest to a region on the genome. It is accepted that a LOD score is statistically more significant than a P value. A P value of 0.05 is accepted as significant whereas a LOD score of 3 is considered significant which is equivalent to a P value of 0.001. For this reason LOD scores are used to determine linkage as opposed to P values, where only LOD scores of 3 and above are considered as indicating significant linkage. This means that the likelihood of linkage occurring is 1000 times greater than no linkage to a region on the genome (Lander and Kruglyak, 1995).

There are four steps involved in the calculation of LOD scores :

1) $\dfrac{\text{Total number of progeny (TP)}}{\text{Number of recombinant progeny (RP) for each marker}}$ = Recombination frequency (RF)

2) $\dfrac{1 - RF}{2}$ = A

3) $\dfrac{(A)^{*NRP} \times (B)^{RP}}{(0.25)^{TP}}$ = C

4) The log of the value found for C will give the LOD score

The values of A and B must add up to 0.5, i.e. 0.5 – A will give value for B. When two genes are unlinked the recombination frequency is 0.5. Therefore, the probability of any given genotype would be 0.25.

*NRP are the non – recombinant progeny i.e. $TP - RP$.

5.2.3 Generation of genetic markers

To create a finer resolution genetic map of chromosome II, more genetic markers needed to be identified. Two approaches were used; firstly, the identification of mini and microsatellite markers and secondly, identification of RFLP markers.

5.2.4 Mini and microsatellite markers

Micro- and minisatellites were identified, by analysing the chromosome II sequence available from Sanger and TIGR with the Tandem repeat finder programme (Benson, 1999). The former were defined as sequences containing >10 copies of a repeat motif of 2-5 nucleotides with >70% sequence identity and the latter were defined using the

same criteria but with a repeat motif of >6 nucleotides. In total 73 microsatellites and nine minisatellites were identified on chromosome II (MacLeod and Tweddie, unpublished results). Primers were designed to unique sequence flanking these candidate markers to generate PCR products distributed across the chromosome. Of the 82 markers 17 were selected for map construction because they showed size variation, and were heterozygous for TREU 927 and/or STIB 386 but homozygous for STIB 247 (El-Sayed *et al.*, 2003) (Fig 5.1). The 17 primer sequences and robocycler conditions for each of the markers are listed in Table 5.1. The PCR amplification conditions were the same as those given in Materials and Methods, Chapter 2. The hybrid progeny used were the 18 previously available and the 20 generated during this study all of which are listed in Table 2.3. The haplotypes for each of these polymorphic markers were determined according to those most frequently inherited in the panel of 38 F1 progeny from the STIB 247 x TREU 927 cross.
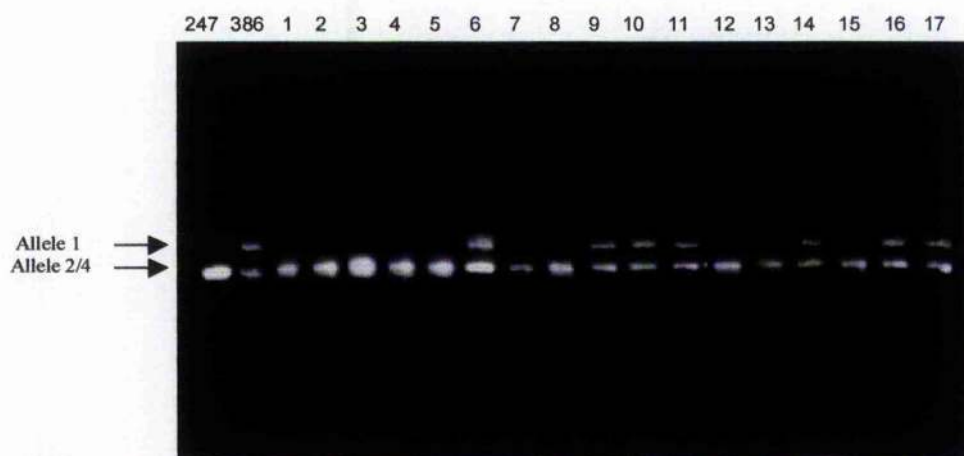
Fig 5.1: PCR amplification of the microsatellite A34, from the parental lines STIB 247 and STIB 386 and 17 hybrid progeny. A34 was one of the 17 markers used for fine scale mapping of chromosome II because it showed size variation, and was heterozygous for STIB 386 but homozygous for STIB 247. Allele scoring and robocycler conditions are listed in Table 5.1.

Table 5.1: List of all oligonucleotide sequences used, running conditions and genotypes for the three parental lines for markers on Chromosome II.

Primer 247 x 927	Designed By	Repeat Type	5´ - 3´ sequence	PCR conditions			Gel type	Genotype 247	Genotype 927
A5 - F	A.M	CAT	ttgaaattgtgtatcggtctg	95°C 50 sec	50°C	65°C 1 min x 30 cycles	3% NuSieve	5 - 5	3 - 4
A5 - R			gggaacagatgtacgatcat						
2 - F	A.T	21 bp	agtagttcctcatttctgcgcagc	95°C 50 sec	54°C 50 sec	70°C 2 min x 30 cycles	2% Seakem	2 - 2	1 - 2
2 - R			gagatcgacgaggagggttcagg						
A8 - F	A.M	AC	tcctctaatcaccgtacct	95°C 50 sec	50°C 50 sec	65°C 1 min x 30 cycles	3% NuSieve	4 - 4	3 - 4
A8 - R			taacatgaagtaactactct						
ST2 - F	S.T	N/A	ggcagaagcagtcagcgaatgtg	95°C 50 sec	56°C 50 sec	65°C 2 min x 30 cycles	2% Seakem	1 - 1	3 - 4
ST2 - R			gatacctgtcttcgcaccgtcacc						
A9 - F	A.M	AT	tgaggcatgaaattgccct	95°C 50 sec	50°C 50 sec	65°C 1 min x 30 cycles	3% NuSieve	4 - 4	3 - 4
A9 - R			ggagatgagggcgtgttcgcg						
A10 - F	A.M	AT	gcgttgggttaagttgagg	95°C 50 sec	50°C 50 sec	65°C 1 min x 30 cycles	3% NuSieve	5 - 5	3 - 4
A10 - R			agacataacaaagacataaatatac						
A34 - F	A.M	AAT	tacacataaattacagcaatatagt	95°C 50 sec	50°C 50 sec	65°C 1 min x 30 cycles	3% NuSieve	4 - 4	3 - 4
A34 - R			cacatattataaccatttgtgga						
5 - F	A.T	ATAAAT	atggcgtgtatcacattcgtgatg	95°C 50 sec	58°C 50 sec	65°C 1 min x 30 cycles	2% Seakem	1 - 1	1 - 2
5 - R			gcgcttcagaagcttaatggcatg						
A12 - F	A.M	AT	ctgtttgggatctcgtgtt	95°C 50 sec	50°C 50 sec	65°C 1 min x 30 cycles	3% NuSieve	4 - 4	3 - 4
A12 - R			ctccctccagcttcttga						
A36 - F	A.M	AAT	aagttacaccagccatagaa	95°C 50 sec	50°C 50 sec	65°C 1 min x 30 cycles	3% NuSieve	5 - 5	3 - 4
A36 - R			atcgctccaccgcaacc						
6 - F	A.T	CT	gttgtgtgagcaacgcaagggcgg	95°C 50 sec	52°C 50 sec	65°C 1 min x 30 cycles	2% Seakem	1 - 1	2 - 3
6 - R			tataacaacaggtgaaggagaggg						

Table 5.1: Cont

Primer	Designed By	Repeat Type	5' - 3' sequence	PCR conditions	Gel type	Genotype 247	Genotype 927
247 x 927							
7 - F	A. T	54bp	acgctaattgtaacctcggctccc	95°C 50 sec 66°C 50 sec 70°C 3 min 65°C x 30 cycles	1% Seakem	1 - 1	2 - 3
7 - R			ccactgatgccgctgagactgtag				
A22 - F	A. M	TTA	ctcgtctctcatcattgct	95°C 50 sec 50°C 50 sec 65°C 1 min x 30 cycles	3% NuSieve	4 - 4	3 - 4
A22 - R			tgagagaatgggattgagg				
X - F	A. T	AC	ccgtaagcgccaactctggtgaag	95°C 50 sec 50°C 50 sec 65°C 1 min x 30 cycles	3% NuSieve	1 - 1	2 - 3
X - R			caggaggtttgttgccgctgcatg				
8 - F	A.M	N/A	gaccgtgcgtcctgctgcgccacc	95°C 50 sec 50°C 50 sec 65°C 1 min x 30 cycles	3% NuSieve	1 - 1	2 - 3
8 - R			gtacaagcgaaggagctgccagac				
A26 - F	A. M	AT	aaggaagaaagtgtagatatga	95°C 50 sec 50°C 50 sec 65°C 1 min x 30 cycles	3% NuSieve	4 - 4	3 - 4
A26 - R			gggtcgcttctttaacataa				
A27 - F	A. M	AT	ctgtgcgtgtaactgtg	95°C 50 sec 50°C 50 sec 65°C 1 min x 30 cycles	3% NuSieve	5 - 5	3 - 4
A27 - R			gaagtgaggacatgcacg				
PLC - G	A. M	AT	caacgacgttggaagagtgtgaac	95°C 50 sec 58°C 50 sec 66°C 50 sec x 27 cycles	3% NuSieve	5 - 5	3 - 4
PLC - H3			ccactgacctttcatttgatcgctttc				
A30 - F	A. M	AT	ctgtgttgcttgttcata	95°C 50 sec 50°C 50 sec 65°C 1 min x 30 cycles	3% NuSieve	5 - 5	3 - 4
A30 - R			agtttaacagcacttccattt				
10C8 I	S. T	N/A	gagtactgccatcagggactcata	95°C 50 sec 56°C 50 sec 65°C 2 min x 30 cycles	2% Seakem	- -	+ -
10C8 J			ggccaatcttccgagagccatccg				
10C8 M	S. T	N/A	ctcctctgcaactttatcagcaag	95°C 50 sec 56°C 50 sec 65°C 2 min x 30 cycles	2% Seakem	- -	+ -
10C8 N			ctcatgaaatcaatcattcaagta				
10C8 Q	S. T	N/A	ctgggacgcaagactgctgataac	95°C 50 sec 56°C 50 sec 65°C 2 min x 30 cycles	2% Seakem	- -	+ -
10C8 R			cgtcatctggttgattggcaccgg				
10C8 U	S. T	N/A	cctctatattggagtaacaccacc	95°C 50 sec 56°C 50 sec 65°C 2 min x 30 cycles	2% Seakem	- -	+ -
10C8 V			cagcagtagcagcaatgacaataa				

Table 5.1 Cont:

Primer	Designed By	Repeat Type	5' - 3' sequence	PCR conditions			Gel type	Genotype 247	386
247 x 386									
A8 - F	A.M	AC	tcctctaatcaccgtacct	95°C	50°C	65°C	3% NuSieve	4 - 4	1 - 2
A8 - R			taacatgaaggtaactactct	50 sec	50 sec	1 min x 30 cycles			
4 - F	A.T	AAAC	cacagatacgaatcttacattgagg	95°C	58°C	65°C	2% Seakem	1 - 1	1 - 2
4 - R			ctgaccgactacggaacagcaccg	50sec	50 sec	2 min x 30 cycles			
A34 - F	A.M	AAT	tacaacataaattacagcaatatagt	95°C	50°C	65°C	3% NuSieve	4 - 4	1 - 2
A34 - R			cacatattataaccatttgtgga	50 sec	50 sec	1 min x 30 cycles			
5 - F	A.T	ATAAAT	atggcgtgtatcacattcgtgatg	95°C	58°C	65°C	2% Seakem	1 - 1	3 - 4
5 - R			gcgcttcagaagcttaatggcatg	50 sec	50 sec	1 min x 30 cycles			
7 - F	A.T	54bp	acgctaattgtaacctcggctccc	95°C	66°C	70°C	1% Seakem	1 - 1	4 - 5
7 - R			ccactgatgccgctgagactgtag	50 sec	50 sec	3 min x 30 cycles			
T - F	A.T	ATT	N/A	95°C	50°C	65°C	3% NuSieve	1 - 1	3 - 4
T - R				50 sec	50 sec	1 min x 30 cycles			
9 - F	A.T	AT	cacggaatctgtcctcgatggtcg	95°C	62°C	70°C	1% Seakem	1 - 1	2 - 4
9 - R			cgagaataggttccatgtatggc	50 sec	50 sec	2 min x 30 cycles			
PLC - G	A.M	AT	caacgacgttggaagagtgtgaac	95°C	58°C	66°C	3% NuSieve	5 - 5	1 - 2
PLC - H3			ccactgacctttcatttgatcgcttc	50 sec	50 sec	50 sec x 27 cycles			

Table 5.1 : List of all the oligonucleotides used for fine scale mapping and linkage analysis on chromosome II. Column 1 lists the name of each primer. Column 2 indicates the name of the person responsible for designing and determining if it was informative (A. M, Annette MacLeod; A. T, Alison Tweedie; S. T, Sonya Taylor). Column 3 shows the type of repeat each primer consisted of, as given by Tandem repeat finder (Benson, 1999) and if a minisatellite (dark shading) or microsatellite. The sequence for each primer, forward (F) and reverse (R), are listed in column 4. The PCR robocyler running conditions and gel type are given in columns 5 and 6. Finally column 7 gives the genotypes for each of the three parental lines, STIB 247, STIB 386 and TREU 927, used to score the alleles inherited by the F1 progeny for the STIB 247 x STIB 386 and STIB 247 x TREU 927 crosses. Also listed are the four RFLP markers used (lightly shaded)The RFLP marker Q/R was digested with the endonuclease Rsa 1, I/J, M/N and U/V were digested with Alu 1. N/A, not available.

5.2.5 PCR – RFLP Markers

After screening for informative mini and microsatellite markers on chromosome II was exhausted, the determination of informative RFLP markers was undertaken. In total 30 sets of candidate RFLP markers were designed from BAC 10C8 on chromosome II that lay between the polymorphic markers 2 and 6 (See Below). Primer pairs were designed approximately 5 kb apart with an average PCR fragment size of 1 kb.

Restriction fragment length polymorphisms (RFLP) that showed variation between the parental lines, STIB 247 and TREU 927 were identified from PCR amplified DNA digested with the restriction enzymes Rsa 1 or Alu 1 (New England Bio-Lab) (Table 5.1). DNA was prepared from the 38 F1 progeny by the method given in Chapter 2. After PCR amplification of DNA, using the selected sets of primers (Robocyler conditions: 95°C for 50 sec, 56°C for 50 sec and 65°C for 2 mins), 2 µl of the product was digested with 2 µl of the selected endonuclease, 2 µl of buffer, NEB 1 and NEB 2 respectively as recommended by the supplier, and 14 µl of sterile water. This 20 µl reaction mix was left to digest overnight in a waterbath at 37°C. Then the 20 µl digestion reactions were separated by electrophoresis on 2% Seakem agarose (20 cm x 20 cm), stained with 0.5 µg/ml ethidium bromide and visualized by UV illumination.

5.2.6 Construction of a genetic map

The genetic map of chromosome II was generated by determining the inheritance of TREU 927 alleles into the panel of 38 F1 progeny from the STIB 247 x TREU 927 cross. To do this, the parental haplotypes were determined on the basis of the two most

frequently inherited haplotypes and the positions of the crossover events were then identified from the more rare recombinant haplotypes. Each of the hybrid progeny was genotyped by PCR amplification of each locus and separation of the products by agarose gel electrophoresis using the conditions listed in Table 5.1. A. MacLeod and A. Tweddie undertook most of the screening of the hybrid progeny with the polymorphic markers leading to the construction of the genetic map.

5.2.7 Genetic Map

Centimorgan (cM) distance was calculated from the observed number of crossover events between each pair of marker pairs divided by the total number of crossovers and multiplying by 100.

5.2.8 Calculations

Chi squared test (χ^2) to determine if the number of crossover events were randomly distributed and if there were any recombination Hot or Cold spots between markers.

$$\chi^2 = \Sigma \ \frac{(O-E)^2}{E}$$

Σ = Sum of
O = observed number of crossovers
E = expected number of crossovers

The expected number of crossovers were calculated by:

Total number of crossover events divided by the total length of the chromosome covered by markers (giving the average number of crossovers per kb) then multiplied by the distance (kb) between each pair of markers.

5.3 RESULTS

5.3.1 Linkage Analysis

At the time of initiating the linkage analysis, genetic maps were available for chromosomes I and II and partial maps were available for chromosomes III, IV, IX and X (Hall *et al.*, 2003; El-Sayed *et al.*, 2003; MacLeod and Tweedie, unpublished results). Linkage was assessed by comparing the inheritance pattern of each marker with inheritance of the cymelarsan resistance phenotype for the 38 F1 progeny of the STIB 247 x TREU 927 cross. Table 5.2 indicates the number of markers initially available for each of the chromosomes and the range of their respective LOD scores for linkage to the drug resistance phenotype. This indicated that only those markers on chromosome II detected linkage to the cymelarsan phenotype, with a highest LOD score of 6.4 for marker 5, which is classified as significant linkage (Lander and Krugylak 1995). One other marker (6) gave LOD scores >3 further confirming the linkage to a region of chromosome II. The remaining markers on this chromosome (A5, 2, 7 and PLC) gave lower LOD scores and, given the physical order of these markers (A5 – 2 – 5 – 6 – 7 – PLC) suggested that the Cymelarsan locus was located in a central region of the chromosome. The markers on the other chromosomes analysed showed no significant linkage, giving LOD score values of <3.

Chromosome	Markers	n	LOD score
I		12	0 – 0.75
II	A5		2.5
	2		2.5
	5		6.4
	6		3.1
	7		2.5
	PLC		0.1
III		16	0 – 0.5
IV		19	0 – 1.08
IX		17	0 – 1.5
X		12	0 – 0.14

Table 5.2: Linkage analysis of Cymelarsan resistance to markers on genetic maps for six of the eleven chromosomes. Statistically significant linkage was observed for a region on chromosome II with a maximum LOD score of 6.4. No significant linkage was observed with markers on any other chromosomes. N = number of polymorphic markers.

The parental haplotypes for each of the 17 polymorphic markers, listed in Table 5.1, were determined for TREU 927 using each pair of markers according to those most frequently inherited in the panel of 38 F1 progeny from the STIB 247 x TREU 927 cross (Fig 5.1). A colour was then assigned for each parental haplotype depending on which pairs of TREU 927 alleles were inherited in the F1 progeny (Fig 5.2) and the association between the haplotypes and the phenotype of the progeny defined. The haplotypes indicated in yellow in Fig 5.2 were associated with resistance while those indicated in blue associated with sensitivity.

By determining the inheritance of phenotype with crossover events in the recombinant progeny an association could be pinpointed to a region on chromosome II. It follows, therefore, that if there was 100% linkage to a pair of markers on chromosome II then all the cymelarsan resistant progeny should be yellow and all the sensitive progeny should

be blue. As can be seen in Fig 5.3 crossover events in the progeny defined the region bounded by markers 2 and 6 as carrying the cymelarsan resistant trait i.e. polymorphic markers that are linked to a segment of the genome should co–segregate with phenotype, with marker 5 giving a LOD score of 6.4 (Table 5.2). As it could not be determined whether the gene conferring the resistance trait was to the left or right of marker 5, fine scale mapping of the region bounded by markers 2 and 6 was undertaken.
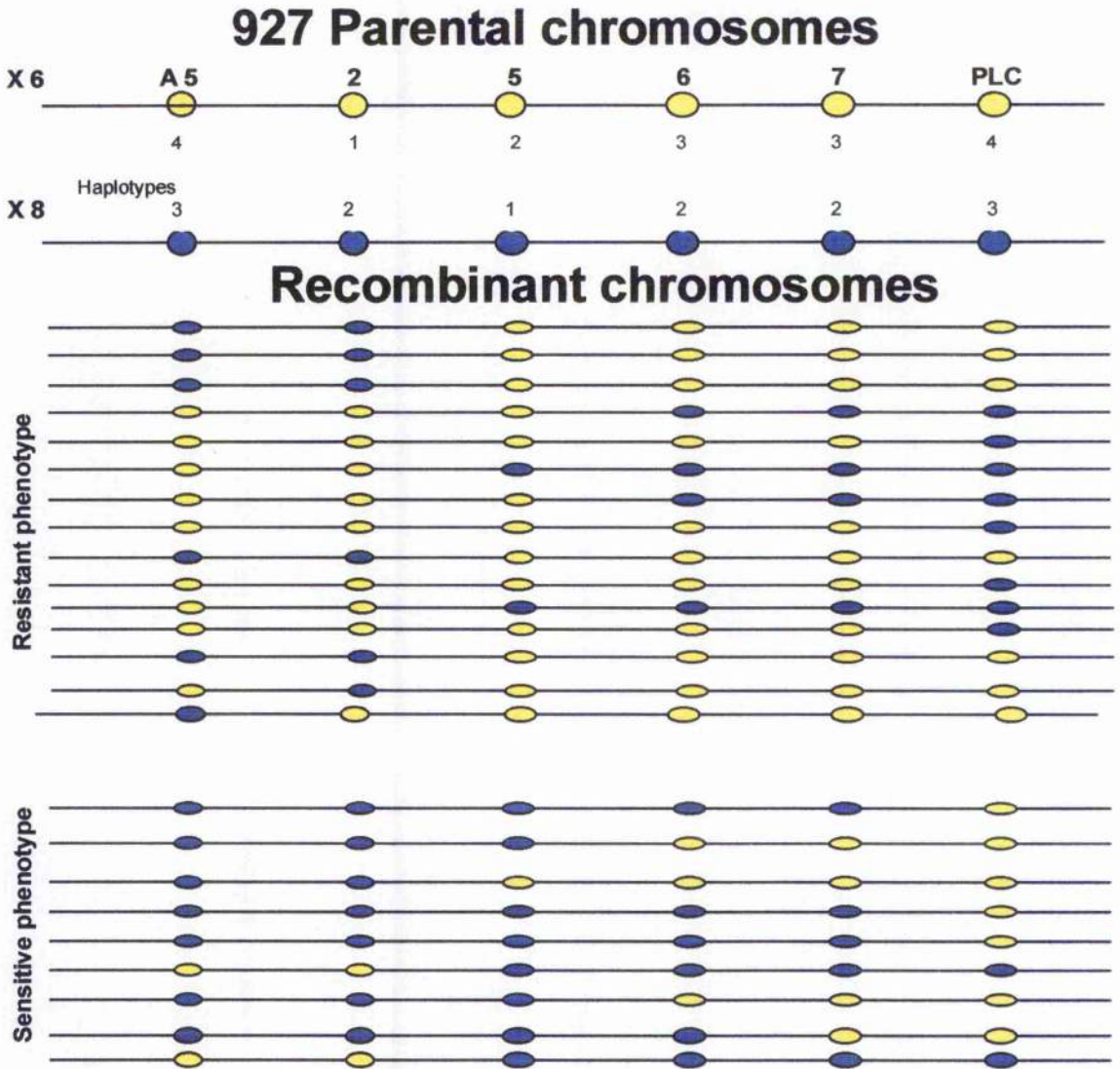
Fig 5.2: Recombination diagram of the haplotypes of TREU 927. Shown are 14 parental haplotypes in blue or yellow and 24 recombinant types. The figure also shows the relationship between genotype and phenotype (yellow, resistant haplotype: blue, sensitive haplotype). Initial findings suggest that the resistance determinant lies between markers 2 and 6. See Fig 5.3 for a more comprehensive analysis.
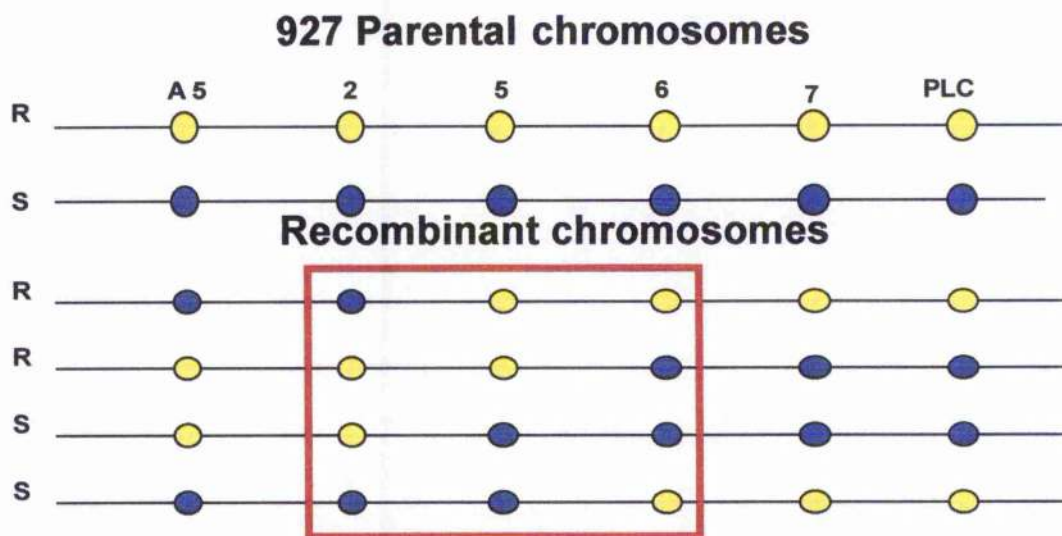
Fig 5.3: Recombination diagram showing the region of chromosome carrying the resistance determinant. Polymorphic marker inheritance patterns and cymelarsan responses (R, resistant and S, sensitive) are shown for four of the progeny from the STIB 247 x TREU 927 cross. The polymorphisms that detected chromosome crossovers are represented are represented with a red box (Yellow, resistant haplotype, Blue, sensitive haplotype). Crossovers occurred between the region bounded by the markers 2 and 6.

As chromosome II has been sequenced (El-Sayed *et al.*, 2003) the distance between markers 2 and 6 was known (Fig 5.4 A) together with the number, order and identity of the open reading frames (ORFs) in this region. The region between markers 2 and 6 was a 263kb segment of chromosome II, containing 50 ORFs.

With the parental haplotypes determined and the positions of crossover events identified in the recombinant haplotypes a genetic map of chromosome II was constructed (Fig 5.4 B). The genetic map was 100 cM (centimorgan) in length, which gave an average of one marker per 16.6 cM. This map so far only covered 738 kb of the 1.2 Mb length of chromosome II, giving an average physical size for the recombination unit of 7.38 kb/cM. Thus, suggesting that the identification of additional crossover

events in the progeny would localise the resistance determinant to a smaller region of the 263 kb segment. Genetic markers had been defined every 123 kb along the chromosome, however a comparison between the genetic and physical maps indicated that recombination was unevenly distributed along its length (χ^2 =11, df = 4, 0.05 > P > 0.02; taking the null hypothesis that there is an equal probability of crossovers in all regions of the chromosome). The chi squared (χ^2) value was determined by using the formula shown in the methods section and adding the values for each pair of markers together. As can be seen from Fig 5.2 there were 15 crossover events between markers 2 and 6 where linkage to the resistance determinant lies. In accordance with this map, however, there should have been approximately one crossover every 30 kb. It was determined, however, that there was a recombination 'hot spot' between markers 2 and 5 where crossovers occurred readily, with 11 observed crossovers in comparison to the 5 expected within a 185 kb region (Table 5.3).

Given the availability of the physical map of chromosome II, markers could be developed within the 263 kb region between markers 2 and 6. Thus, this fine scale mapping should localise the resistance determinant to a smaller region of the chromosome and as a result cut down the number of genes from 50, hopefully to a more workable number. Therefore fine scale mapping of this 263 kb region was undertaken which would also improve the resolution of the genetic map of chromosome II. It should also be noted that some informative makers were also found outside the 263 kb region of interest which subsequently led to improve the genetic map.

Chromosome II Genetic Map for TREU 927

A

| 0kb | 100 | 200 | 300 | 400 | 500 | 600 | 700 | 800 | 900 | 1Mb | 1.1 | 1.2 |

A5 2   5   6 7   PLC

B

46	185	78	49	379	kb
8	44	16	4	28	cM
2	11	4	1	7	No of crossovers
5.8	4.2	4.8	12.25	13.5	kb/cM
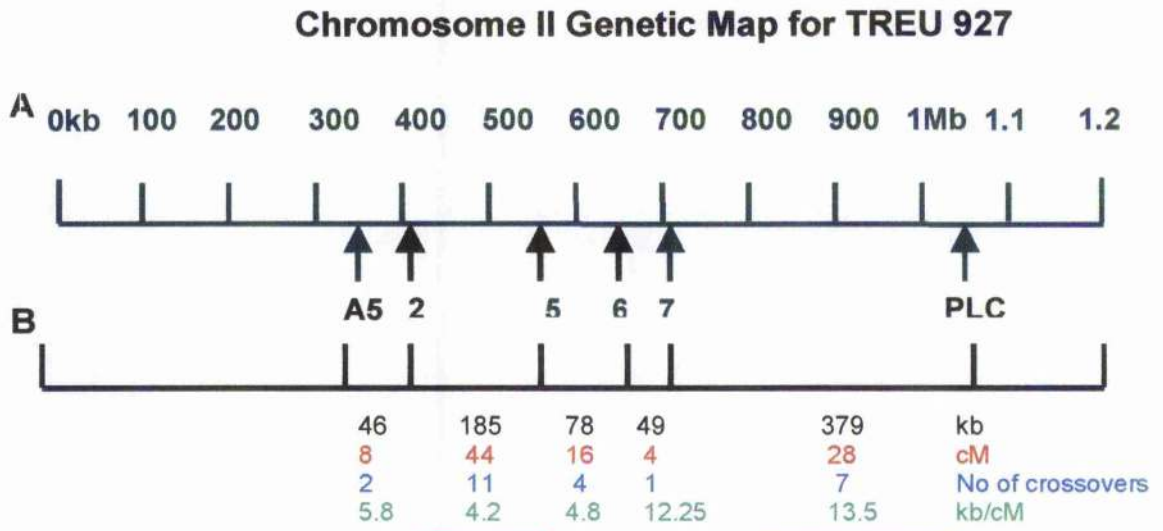
Fig 5.4 : Position of polymorphic markers on chromosome II and a genetic map. (A) The location of the polymorphic markers along chromosome II. **(B)** Genetic map showing the physical distances between the markers (kb), and the genetic distances (cM) calculated from the recombination frequencies (see Materials and Methods). The relationship between hot and cold spots of recombination (kb/cM) are also indicated (Hot, red line, Cold, blue line), see also Table 5.3.

Marker Pairs on Chromosome II	Distance between Markers (kb)	Number of Crossovers	
		Observed	Expected
A5 & 2	46.3	2	2.7
2 & 5	185	11	5
5 & 6	78	4	2.3
6 & 7	49	1	1.4
7 & PLC	379	7	12

Table 5.3 : Indicating hot and cold spots of recombination. There is recombination 'hot spot' between markers 2 and 5 where crossovers occur readily, i.e. exceed the number expected, (2.6 kb/cM) (red). In contrast, there is a recombination 'cold spot' between markers 7 and PLC where there were fewer crossovers than expected over the 379 kb region (blue). $\chi^2 = 11$, df = 4, $0.05 > P > 0.02$, indicating crossovers do not occur randomly. The methods for these calculations are shown in Materials and Methods.

Ten mini- and microsatellite informative polymorphic markers were identified (Table 5.1) which were heterozygous for the TREU 927 parental line used in the cross (Fig 5.1). Thirty sets of primers were designed and used to amplify genomic DNA from STIB 247 and TREU 927. Each amplicon was then digested with either Alu 1 or Rsa 1 to screen for polymorphisms between the two parental lines. Only four were found to be informative because they showed variation between the two parental lines (Table 5.1). As with the mini and microsatellite markers, the parental haplotypes of the four RFLP markers were determined as the most frequently inherited haplotypes (presence of band (+), absence of band (-)) for the panel of 38 F1 progeny of the STIB 247 x TREU 927 cross (Fig 5.5).
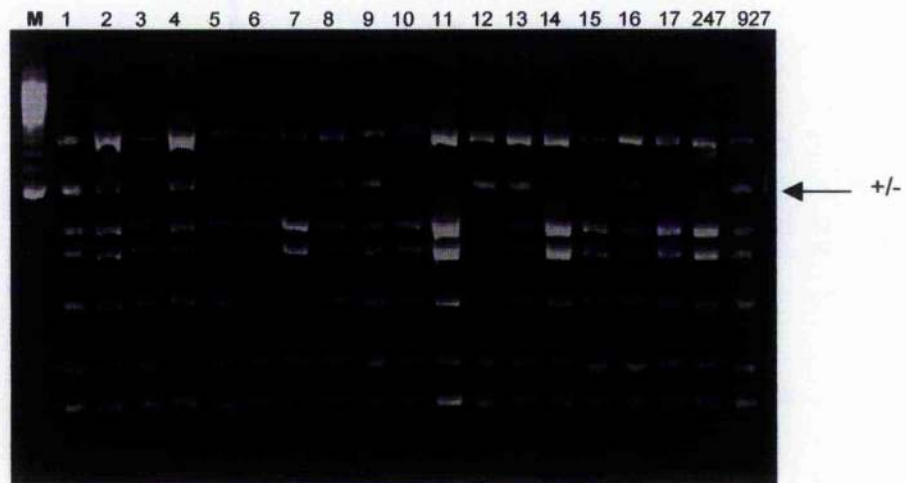
Fig 5.5: The RFLP inheritance pattern of the 2 parental lines (247 & 927) and 17 hybrid progeny digested with restriction enzymes, located on chromosome II. The RFLP marker M/N was scored after digestion with the restriction endonuclease Alu1, indicating a polymorphism in the restriction fragment (arrowed) that is present in TREU 927 but absent in STIB 247. This polymorphism segregated into the F1 progeny as shown by the presence of the band in 9 of the 17 progeny. Robocycler conditions are listed in Table 5.1, the PCR product was digested overnight at 37°C and separated by electrophoresis on 2% Seakem agarose stained with ethidium bromide and visualized by UV illumination. M, 100 bp DNA ladder.

Eleven of the 14 informative markers identified were then used to define a genetic map within the 263 kb region where the resistance determinant was thought to be located (Fig 5.6). Inheritance patterns of these markers in the 38 F1 recombinant progeny identified 27 crossover events across the chromosome (Fig 5.7). The boundaries set by these crossovers, therefore, defined the locus determining the Cymelarsan phenotype.
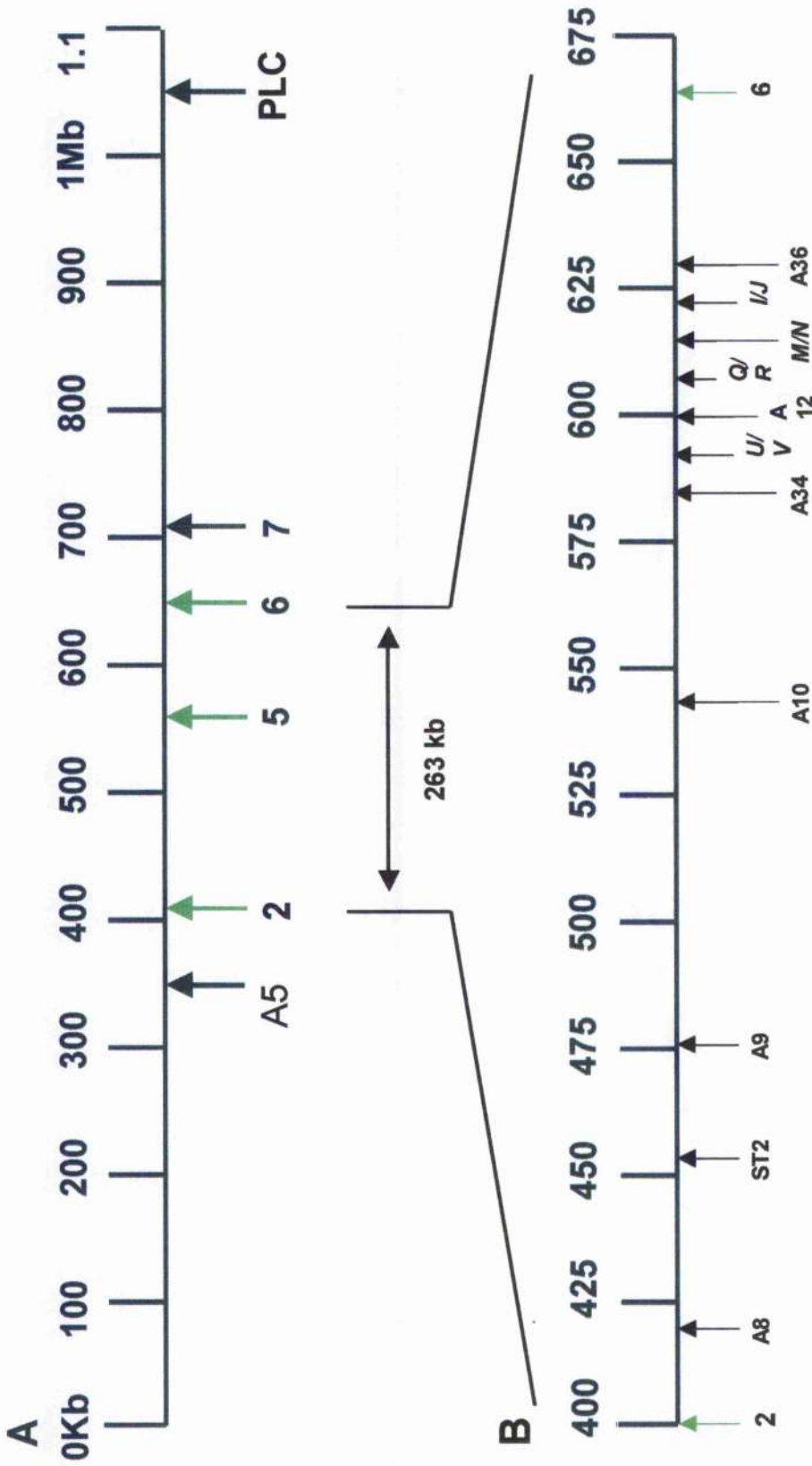
Fig 5.6: Map of chromosome II region carrying the determinant of the MelCy^R phenotype in the 247 x 927 cross. (A) The relative positions of the polymorphic markers are shown along the chromosome line. There was suggestive linkage to the MelCy^R phenotype. **(B)** Fine grained mapping within the initially identified 263 kb region where this yielded a further 11 informative markers which were then used to narrow the region of linkage of the resistance phenotype in the progeny of the 247 x 927 cross. RFLP markers are indicated in italics.

141
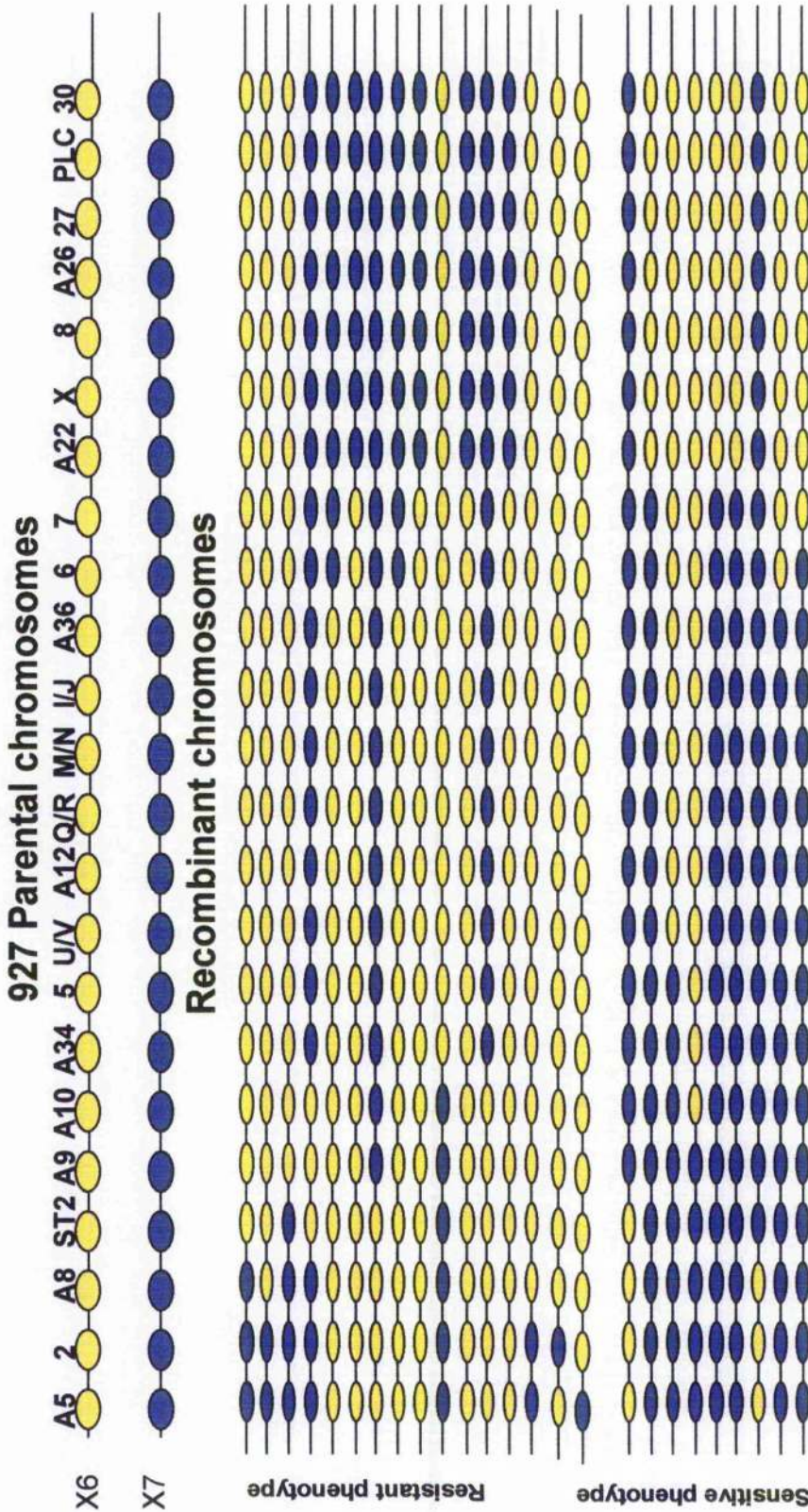
Fig 5.7: Recombination diagram showing crossover events in the resistant and sensitive progeny of the STIB 247 x TREU 927 cross. There were 23 informative markers on chromosome II. Genotype analysis of the 38 F1 progeny revealed that 25/38 F1 progeny were recombinant. Also indicated is the linkage relationship between genotype and phenotype (Yellow, Resistant haplotype; Blue, Sensitive haplotype). Initial findings suggested that the resistance determinant is located between markers ST2 and A9, this will be further discussed later in the chapter and in Fig 5.9.

Using the same approach as that previously described for assessing linkage (Fig 5.3), analysis revealed that alleles of the ST2 and A9 markers were highly significantly linked to the cymelarsan resistance phenotype, giving a LOD score of 7.5. To either side of these markers, linkage ratios were reduced due to chromosome crossover events among the progeny and Fig 5.8 shows a plot of the LOD scores for all the markers across the length of chromosome II. From this data a region of the chromosome, between markers A34 and A36, gives LOD scores that are >3. However, it should be noted that the distance between these markers is 41 kb and so few crossover events would be predicted within this region. This is discussed in more detail later in the chapter (See also Fig 5.11).

The positions of crossovers in three of the most informative progeny clones are indicated in Fig 5.9. The phenotype of these three hybrid clones was identical to that of either the resistant parental line TREU 927 or the sensitive parental line STIB 247. The haplotypes of these clones define crossovers between markers ST2 and A9 and the phenotypes of these clones can only be explained if the locus determining Cymelarsan resistance lies between these markers. The availability of the entire TREU 927 sequence of chromosome II from TIGR identified this region as spanning 25 kb and contained six ORFs with the linkage and haplotypes analysis indicating one of these genes as determining resistance to cymelarsan (Fig 5.10).
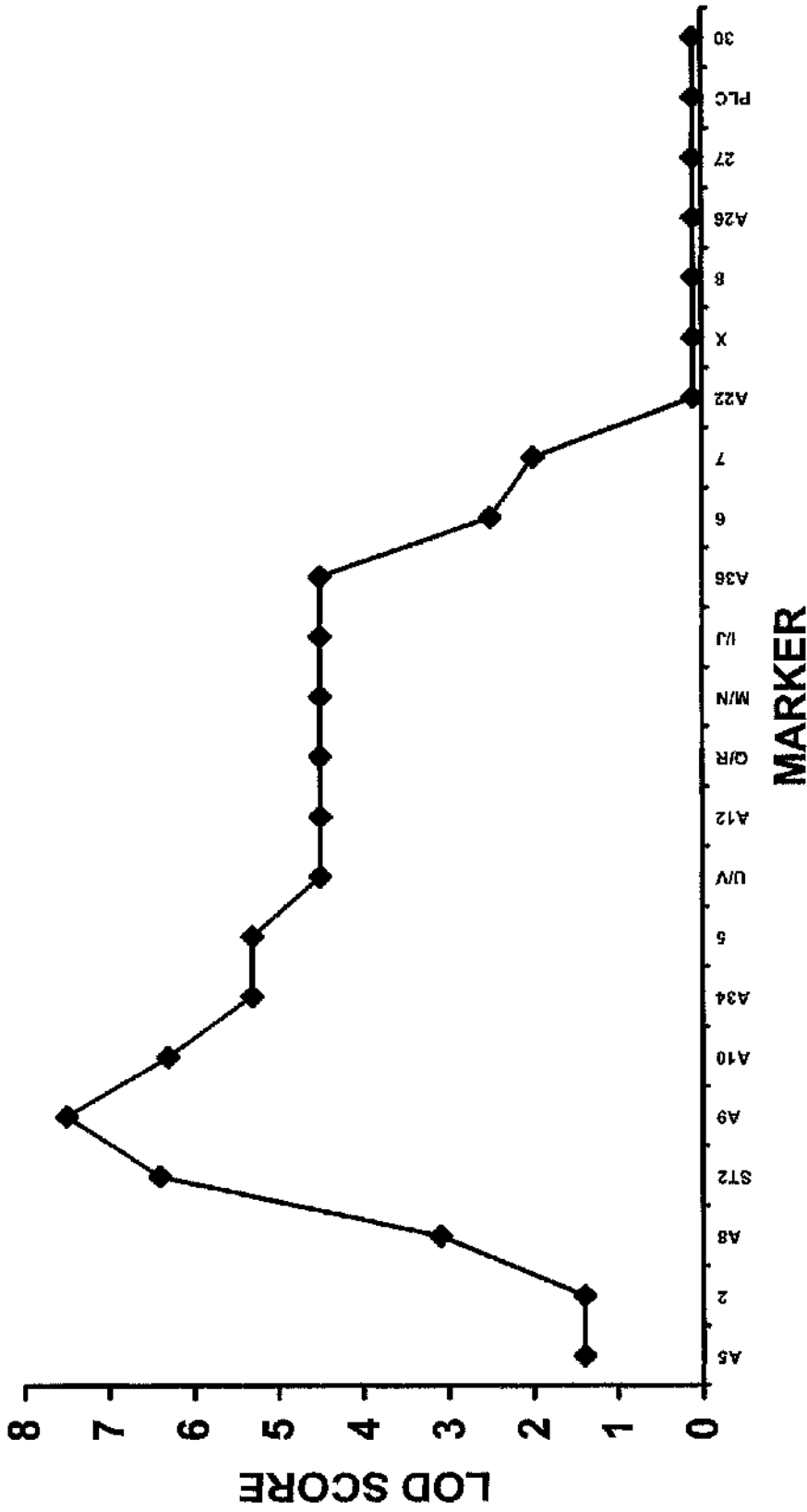
Fig 5. 8: LOD scores indicating the region on Chromosome II where linkage to the resistance determinant may lie. The results indicated that the resistance determinant may lie between markers ST2 and A9 with a LOD score of 7.5.To either side of these markers linkage ratios were decreased due to chromosomal crossover events among the progeny.
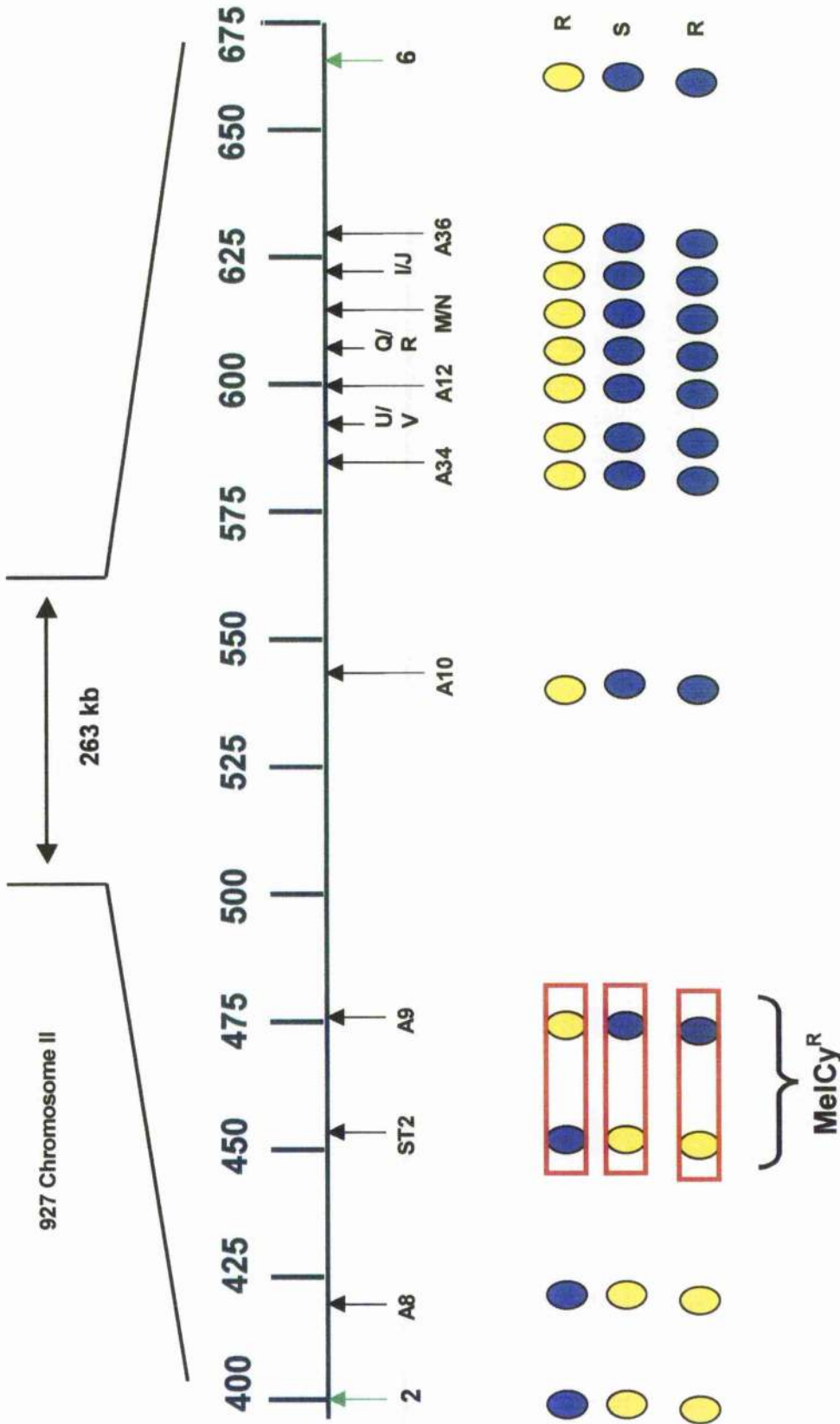
Fig 5. 9: Map of the region on chromosome II carrying the determinant of the MelCy^R phenotype in the STIB 247 x TREU 927 cross. Polymorphic marker inheritance patterns and cymelarsan responses (R, resistant and S, sensitive) are listed for 3 of the progeny from the 247 x 927 cross. The polymorphisms that detected chromosome crossovers are represented with red boxes (Yellow, resistant haplotype; Blue, sensitive haplotype). Crossovers bound the 25 kb region containing the putative MelCyR determinant between markers ST2 and A9 in three of the clones represented here with solid red lined box.

145

Fig 5.10: Schematic representation of all the ORFs, their direction and predicted functions on chromosome II (El-Sayed *et al.*, 2003). Those circled in red are the 6 ORFs between markers ST2 and A9, identified by positional cloning, one or more of which are thought to be involved in conferring the MelCy resistance phenotype, which will be discussed further in Chapter 6.

5.3.2 Genetic map of TREU 927 for chromosome II

As a result of generating a further 14 informative polymorphic markers on chromosome II in addition to the nine already described (El-Sayed *et al.*, 2003; MacLeod and Tweddie, personal communication) and increasing the number of independent progeny clones from 18 to 38, it was possible to construct a more detailed genetic map of TREU 927. By calculating the recombination fraction (cM) between each pair of markers along the chromosome and adding these together the *T. brucei* genetic map of chromosome II was 97 cM in length, which gave an average density of approximately one marker per 4.2 cM. The map covers 738 kb of chromosome II, which gives an average physical size for a recombination unit of 7.6 kb/cM (Fig 5.11). Thus on average, a crossover event occurs every 27 kb along the chromosome II but comparison of the genetic map and the physical map indicated that the genetic distance varied in relation to the physical distance between markers. In order to determine if recombination events were evenly distributed along the chromosome, firstly the observed and expected number of crossover was determined for each interval between the markers based on the average for the whole chromosome (Materials and Methods, section 5.2.8) (Table 5.4). As some of the physical intervals between markers were small and therefore the predicted number of crossovers less than expected, the intervals were combined to yield 12 contiguous pairs of markers between each of which the predicted number of crossovers was greater than one. Based on the predicted versus observed numbers of crossovers (Table 5.5) in each of the intervals and testing the deviation between these using χ^2, it can be seen that two marker intervals show significant differences between observed and expected values. There was one recombination 'hot spot' between markers 2 and

A9 where crossovers occurred readily, with 9 observed crossovers in comparison to the 2.6 expected value within a 77 kb region (2.3 kb/cM) and one 'cold spot' between markers A22 and A30 where crossovers were absent over a 269 kb region (>269 kb/cM) but 9.7 crossovers are predicted (Table 5.5) and (Fig 5.11).

Markers on Chromosome II	Distance between Markers (kb)	Number of Crossovers	
		Observed	Expected
A5 &2	46	2	1.6
2 & A8	24	3	0.8
A8 & ST2	27	3	0.9
ST2 & A9	26	3	0.9
A9 & A10	66	1	2.3
A10 & A34	41	3	1.5
A34 & 5	1	0	0.03
5 & U/V	0.3	1	0.01
U/V & A12	7	0	0.2
A12 & Q/R	1.5	0	0.05
Q/R & M/N	7.5	0	0.27
M/N & I/J	9.5	0	0.3
I/J & A36	14	0	0.5
A36 & 6	38	3	1.4
6 & 7	49	1	1.7
7 & A22	121	7	4.3
A22 & X	39	0	1.4
X & 8	44	0	1.6
8 & A26	129	0	4.6
A26 & 27	37	0	1.4
27 & PLC	9	0	0.3
PLC & 30	11	0	0.4

Table 5.4: Markers, their position, the distance (kb) and the number of crossovers, observed and expected, between each of the markers, for the 247 x 927 cross, on chromosome II. These were then used to determine if there were any recombination 'hot spots' or 'cold spots' on the chromosome. Those with the lowest expected values were merged to raise the expected values to 1 or more (Table 5.5). The observed number of crossovers was obtained from Fig 5.7. See Materials and Methods for calculation of expected number of crossovers for each marker pair.

Marker Pairs on Chromosome II	Distance between Markers (kb)	Number of Crossovers		χ^2
		Observed	Expected	
A5 & 2	46	2	1.6	0.1
2 & A9	77	9	2.6	15.7
A9 & A10	66	1	2.3	0.73
A10 & A34	41	3	1.5	1.5
A34 & A36	40.8	1	1.36	0.95
A36 & 6	38	3	1.4	1.8
6 & 7	49	1	1.7	0.29
7 & A22	121	7	4.3	1.7
A22 & X	39	0	1.4	1.4
X & 8	44	0	1.6	1.6
8 & A26	129	0	4.6	4.6
A26 & 30	57	0	2.1	2.1
				32.47

Table 5.5: Isolation of 'hot and cold spots' on Chromosome II. After the merging of several groups of adjacent markers it was observed that there was one recombination 'hot spot' between markers 2 and A9, where there were 9 observed crossover events compared with the expected value of 2.6 over a 77 kb region (2.3 kb/cM)(red) and one 'cold spot' between markers A22 and 30 where there were no observed crossovers. The expected value was ~ 10 over a 269 kb region (>269 kb/cM) (blue). $\chi^2 = 32.47$, df = 10 (cumulative data set) and P< 0.01, indicating crossovers do not occur randomly.

Chromosome II Genetic Map for TREU 927

Marker positions (kb): 0 kb, 353, 400, 424, 451, 476, 543, 584, 585, 586, 592, 594, 601, 611, 625, 663, 712, 833, 872, 916, 1044, 1082, 1091, 1102

Marker names: A5, 2, A8, ST2, A9, A10, A34, 5, U/V, A12, Q/R, M/N, I/J, A36, 6, 7, A22, X, 8, A26, 27, PLC, 30

kb	46	24	27	26	66	41	1	0.3	7	1.5	7.5	9.5	14	38	49	121	39	44	129	37	9	11
cM	7	11	11	3	11	0	3	0	0	0	0	11	3	26	17	0	0	0	0	0	0	0
No of crossovers	2	3	3	1	3	0	1	0	0	0	0	3	1	7	0	0	0	0	0	0	0	0
kb/cM	6.6	2.2	2.4	2.3	22	3.7	0	0.1	0	0	0	3.4	16	4.6	0	0	0	0	0	0	0	0

2.3 kb/cM   > 269 kb/cM
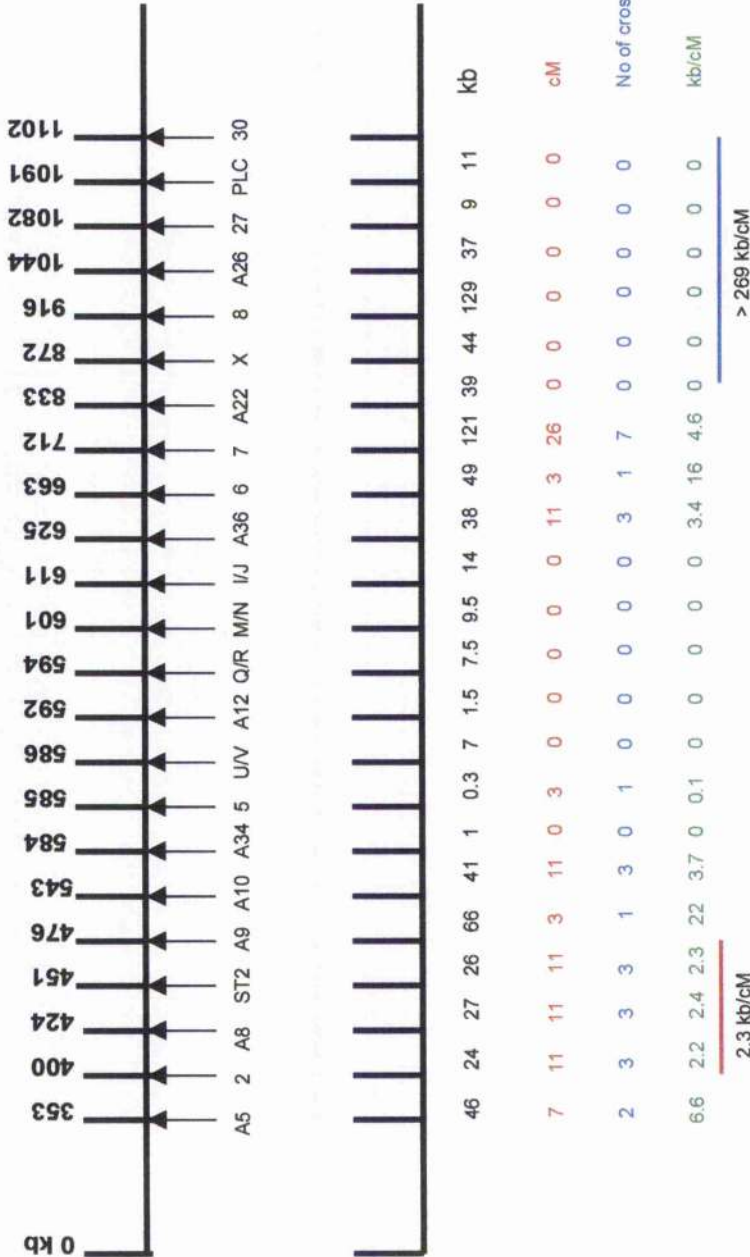
Fig 5.11: Genetic map of chromosome II for TREU 927. The data was obtained from the cross between STIB 247 x TREU 927 indicating the polymorphic markers used in the analysis. The number of crossover events which the genetic distance (cM; red)) was derived, are indicated in blue and also the physical size of the recombination unit (kb/cM; green) based on the distance between markers from the TREU 927 sequence. The relationship between hot and cold spots of recombination are also shown in kb/cM (red line 'hot spot', blue line 'cold spot').

5.3.3 Analysis of the STIB 247 x STIB 386 cross

The next step of the analysis was to determine if cymelarsan resistance mapped to the same region in STIB 386. To date, genetic maps of STIB 386 are only available for chromosomes I and II and mapping of the remaining chromosomes is in progress (MacLeod and Tweedie, personal communication). Due to the fact that the STIB 386 genome has not been sequenced, markers for this line were developed based on the TREU 927 sequence (El-Sayed *et al.*, 2003). The same 82 markers described previously for screening TREU 927 (MacLeod and Tweedie, unpublished results), were screened using STIB 386 and STIB 247 to determine if they were informative (Materials and Methods). I screened 28 of these markers and the remaining 54 were screened by A. MacLeod and A. Tweedie and eight were selected for map construction because they showed size variation, and were heterozygous for STIB 386 but homozygous for STIB 247 for chromosome II (Fig 5.1; Table 5.1), similarly there were seven informative markers available for chromosome I (MacLeod and Tweedie, unpublished results).

Linkage was assessed by comparing the inheritance pattern of each marker with inheritance of the cymelarsan resistance phenotype for the 34 F1 progeny of the STIB 247 x STIB 386 cross. Table 5.6 presents the number of markers analysed for each of the chromosomes and the range of their respective LOD scores for linkage to the drug resistance phenotype. This indicated that only markers on chromosome II showed linkage to the cymelarsan phenotype, with a LOD score of 4.1 for markers 4, A34, 5 and 7, which is classified as significant linkage (Fig 5.12).

As there are, to date, only eight markers available for mapping the resistance determinant for this cross, it is difficult to say that the resistance determinant maps to the exact region as that identified for the STIB 247 x TREU 927 cross, but these results suggest that the locus is at the same end of the chromosome (Fig 5.12).

Chromosome	Markers	LOD score
I	10	0.2 – 0.41
II	A8	1.7
	4	4.1
	A34	4.1
	5	4.1
	7	4.1
	T	2.7
	9	2.7
	PLC	1.7

Table 5.6: Linkage analysis of Cymelarsan resistance to markers on genetic maps for chromosomes I and II of STIB 386. Statistically significant linkage was observed for a region on chromosome II with a maximum LOD score of 4.1. No significant linkage was observed with markers on chromosome I.
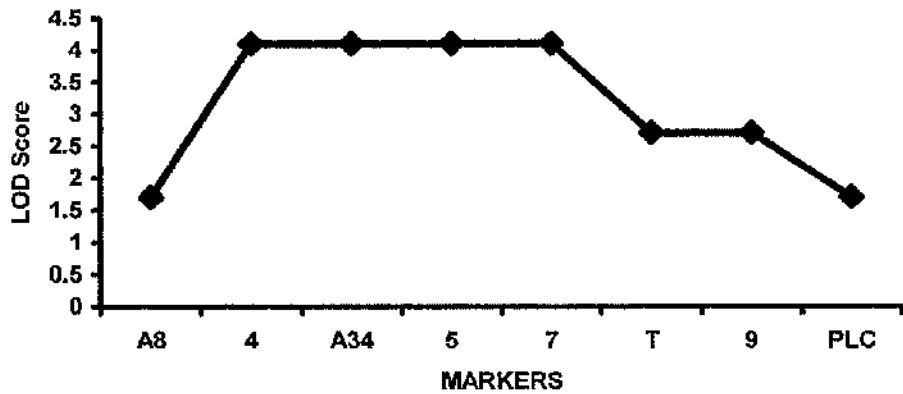
Fig 5.12: LOD scores for markers situated along chromosome II of STIB 386. Graph indicating the region on Chromosome II where linkage to the resistance determinant lies between markers 4 and 7 with a LOD score of 4.1. To either side of these markers linkage ratios were decreased due to crossover events among the progeny.

The haplotypes of each of the markers on chromosome II were determined according to those most frequently inherited in the panel of 34 F1 progeny from the STIB 247 x STIB 386 cross. Linkage to cymelarsan resistance was determined by analysing the inheritance of genotype with phenotype in the 34 F1 progeny (Fig 5.13). The polymorphic markers, therefore, that link to a segment in the genome should co-segregate with phenotype (Blue, resistant, Red, sensitive). Thus, a crossover defined interval carrying the gene controlling the inheritance of the cymelarsan resistance trait can be localized to a region on the chromosome.

Taking together, the STIB 386 haplotypes of the 34 F1 progeny clones and their co-segregation with phenotype, crossover events localised the resistance determinant between markers A8 and 4 (Fig 5.13). Taking this into account and together with the

LOD scores available future work should focus on fine grained mapping between markers A8 and 4 in order to confirm the exact position of the resistance determinant on chromosome II and thus ascertain if it is in the same region as that mapped in line TREU 927.
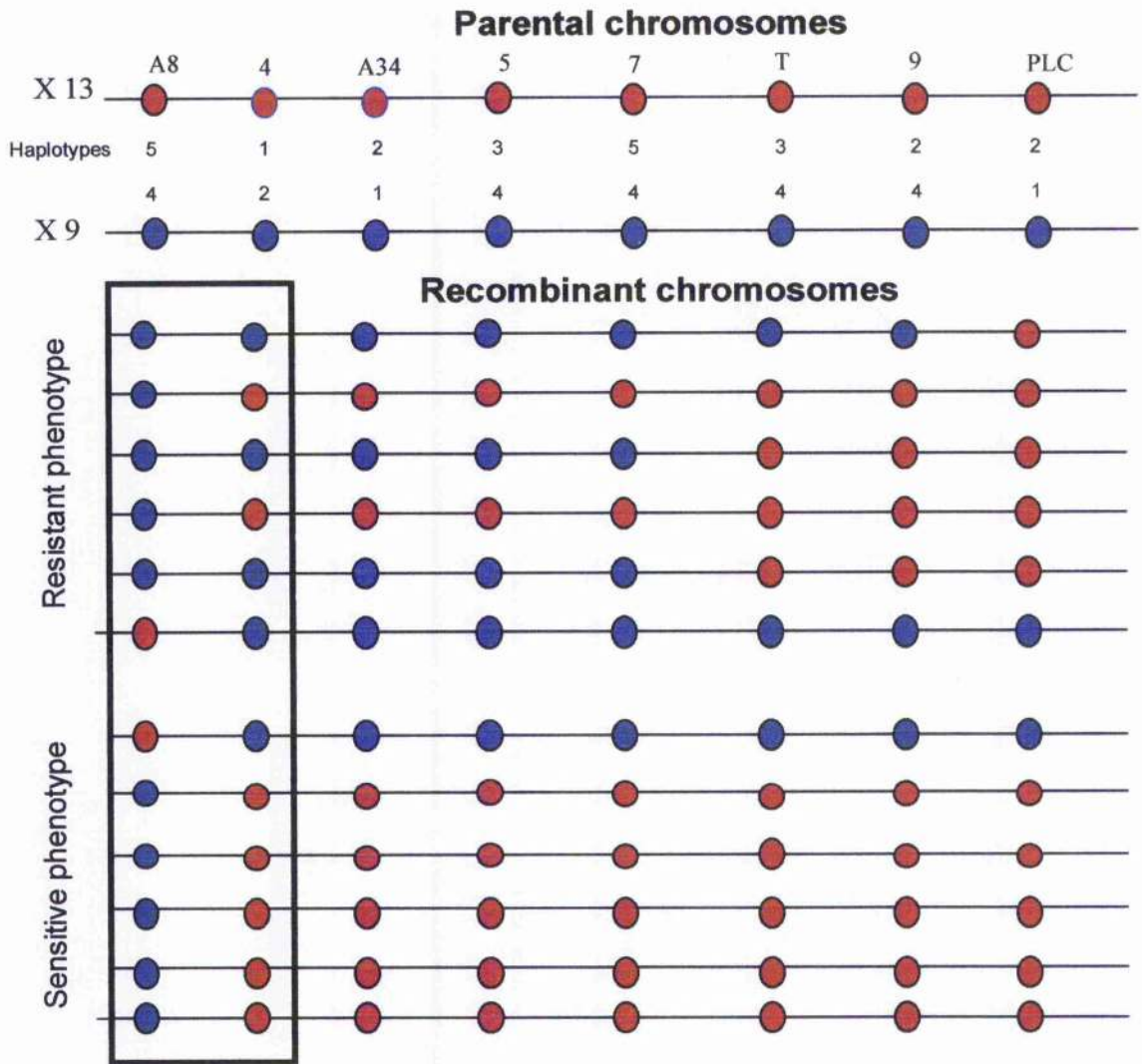
Fig 5.13: Recombination diagram of the haplotypes of STIB 386. Shown are 22 parental haplotypes in blue or red and 12 recombinant types. The figure also shows the relationship between genotype and phenotype (blue, resistant haplotype: red, sensitive haplotype). Initial findings suggest that the resistance determinant lies between markers A4 and 4 (Black box).

5.4 DISCUSSION

The availability of a genetic linkage map for *T. brucei* makes it possible to perform classical genetic studies to identify the loci that determine drug resistance, and relies upon the ability to conduct genetic crosses between two parents that differ in their drug resistance phenotype. Partial genetic maps were previously constructed using mini- and microsatellite markers (MacLeod and Tweedie, unpublished results) and anonymous AFLP markers (Masiga *et al.*, 2002; Tait *et al.*, 2002). However, the rapid progression of the *T. brucei* sequencing project has meant that genetic mapping and positional cloning is now feasible, based on mini and microsatellite markers at defined positions on each of the chromosomes. These chromosome–anchored maps provided a baseline for the study described in this chapter.

The ability to perform genetic linkage analysis in *T. brucei* has allowed the investigation of the trait of resistance to the arsenical drug cymelarsan. Analysis of the genotype of the unique hybrid progeny was conducted using mini and microsatellite and RFLP markers. In parallel, each progeny clone was tested for its drug resistance phenotype (Chapter 4). By comparing the segregation of the drug resistance phenotype with that of markers that are located at known map positions along the chromosome it was possible to identify the locus determining drug resistance. More specific, ' fine grained', analysis of the locus was possible by using a combination of further linkage analysis combined with physical mapping facilitated by the genome project for *T. brucei*.

The results indicated that a major determinant of drug resistance was associated with a locus on chromosome II between two polymorphic markers, ST2 and A9, encompassing a 25 kb region containing 6 ORFs, identified by TIGR as 'conserved hypothetical' or '*T. brucei* family hypothetical proteins'.

As the physical distance between the 23 markers on chromosome II is known from the genome sequence (El-Sayed *et al.*, 2003), the physical size of the centimorgan could be calculated for each interval (Fig 5.11). The average value obtained was 7.6 kb/cM for the marker intervals analysed, however, this is probably an underestimate as this map only covers 738 kb of the 1.2 Mb chromosome. This value is comparable to the value obtained for Plasmodium (Su *et al.*, 1999) and thus a 10cM map of the genome would allow linkage to be determined within 110 kb of any gene. Genetic maps are currently being constructed for the *Toxoplasma gondii* genome and the average map unit is estimated to be approximately 215 kb/cM. A locus has been identified on chromosome VII that is thought to be involved in acute virulence, a region that spans 1.5 Mb. The ongoing genome sequencing project of *T. gondii* means that physical mapping of this region to eventually isolate the gene involved in acute virulence is restricted (Su *et al.*, 2002). Similarly, construction of genetic maps for *Eimeria tenella* are in progress. A region on chromosome II is thought to be responsible for precocious development in progeny (faster completion of life cycle stages than their parents), however because the linkage groups where the markers defining this region have not been assigned a position on the chromosome the position of crossovers are not known. Physical

mapping of this trait to a smaller region is not possible until a larger number of chromosome II specific markers are identified (Shirley and Harvey, 2000).

Comparison of the predicted sequences of these six ORFs against the protein database InterProScan (http://www.ebi.ac.uk) revealed sequence homology to ORF 1 with a phospholipase C X – domain, 34 amino acids with an E – value of 0.21. For ORF 2 there was sequence homology to a RING finger domain identifying homology for 34 amino acids of a 103 amino acid sequence and a hypothetical protein that showed sequence homology for 27/67 amino acids. A SMART (http://smart.embl) search of ORF 4 found that there was a transmembrane domain near the N – terminus, which may function to anchor the protein in the membrane. No other sequence homologies were detected for the remaining three ORF protein sequences. Given these data, where there were no outstanding features of any of the ORFs that would implicate their involvement in conferring drug resistance, further investigation of these 6 ORFs is required and this is the subject of the following chapter.

The linkage of the cymelarsan resistance determinant in the STIB 247 x STIB 386 cross was less conclusive. Analysis showed that the resistance determinant was located on chromosome II giving a LOD score of 4.1 between markers 4 and 7 (Table 5.6), which suggested significant linkage of the resistance determinant to this region of chromosome II. Recombination events in the 34 F1 progeny linked the resistance determinant between markers A8 and 4 (Fig 5.13). It, therefore, would be important to carry out fine grained mapping within the 288 kb region between markers A8 and 7 to

further refine the region containing the resistance determinant. It is difficult, without the entire sequence of STIB 386 chromosome II, to determine if the markers are located physically where predicted, due to the fact that these markers were based on the TREU 927 sequence available from the TIGR *T. brucei* sequencing project (El-Sayed *et al.*, 2003). These markers, however, are in the same part of chromosome II where there was highly significant linkage for the resistance determinant found for the STIB 247 x TREU 927 cross.

Fine scale mapping should be a relatively easy task due to the fact that the distance between marker A8 and 4 is 142 kb, in strain TREU 927 and this region contains 22 annotated genes (El-Sayed *et al.*, 2003). With a combination of a genetic map, a genome sequence and phenotypic difference between the parents, localisation to a smaller region is relatively straightforward.

It is likely, however, that there are additional loci contributing to the drug resistance phenotype. For instance the locus/loci which governs the resistance phenotype observed for pentamidine for the STIB 247 x TREU 927 cross for which all the progeny were sensitive to pentamidine in comparison to the progeny from the STIB 247 x STIB 386 cross where the progeny segregated in a 1 : 1 ratio sensitive : resistant which was observed for all other drugs tested for both crosses, suggesting that there is a different locus involved in pentamidine resistance (Chapter 2). Although, this is not a trivial undertaking, this could be resolved by analysis of further progeny from different crosses, linkage analysis and the availability of a backcross. These results, however,

demonstrate that forward genetics offers a powerful means of analysing the genetic basis of drug resistance in *T.brucei*. Similar approaches, therefore, are likely to be useful for determining the molecular genetic basis of other complex traits.

Although trait mapping using laboratory derived progeny is an essential tool for studying the inheritance of particular trypanosome phenotypes, there are limitations to this approach. It is relatively expensive and laborious to generate laboratory crosses and large numbers of progeny are required for analysis of the trait of interest. The RNAi approach, however, is even more laborious and suffers from often not observing a phenotype and therefore the genetic approach for gene identification responsible for a particular trait far outweighs the alternative RNAi approach. The relevance of the loci identified in the selection of drug resistance in the field remains to be determined. It is possible that different alleles at the locus described here and other loci are involved in determining resistance to arsenicals. It should, therefore, be remembered that genetic analysis of laboratory generated progeny from crosses acts principally to guide testing of field isolates for the genes identified.

The process of trait mapping and, ultimately gene identification will dramatically facilitate the ongoing *T. brucei* sequencing effort. Due to the fact, each mini-microsatellite marker in the linkage map corresponds to a sequence tag site (STS) it is possible then that these can be readily identified in the expanding sequencing contigs. As a result, mapped loci can be linked to physical sequences, and specific candidate genes residing within these sequences can be identified and evaluated for their role in trait

determination. Moreover, the sequencing project will continue to identify thousands of simple sequence repeats from which new markers can be identified and used to saturate regions of particular interest in the genome. We should not lose sight of the fact that proof of the role of any candidate gene will be a rigorous task.

CHAPTER 6

DETERMINATION OF A PUTATIVE RESISTANCE GENE USING A PCR BASED ALLELE DELETION AND REPLACEMENT SYSTEM

6.1 INTRODUCTION

Biochemical studies have indicated that the arsenical drug resistance phenotype arises when a plasma membrane nucleoside transporter involved in drug uptake, P2, is altered or lost (Carter and Fairlamb, 1993). The gene encoding the P2 transporter, *TbAT1*, has been identified (Mäser *et al.*, 1999) and is located on *T. brucei* chromosome XI. Knock-out experiments have shown that the absence of this gene confers resistance, but not to the levels obtained in drug selected cell lines (Matovu *et al.*, 2003). The data presented in previous chapters, however, has shown that a locus on chromosome II is a determinant of the drug resistance phenotype (Chapter 5) and therefore resistance can arise by mutations in more than one gene (Chapter 4).

The drug resistance phenotype segregated as a Mendelian trait in each of the two genetic crosses, STIB 247 x TREU 927 and STIB 247 x STIB 386 where TREU 927 and STIB 386 are both drug resistant, relative to STIB 247. Linkage mapping of the STIB 247 x TREU 927 cross localized the drug resistance determinant to a 25 kb region on Chromosome II and a search of this region identified six ORFs (Chapter 5). To further investigate if these six candidate ORFs are determinants of the drug resistance phenotype, a PCR based gene/allele deletion strategy was undertaken.

Up until now this study has focused on and demonstrated that forward genetics is a powerful approach in the analysis of the molecular basis of drug resistance in *T. brucei*. It is obvious, however, that coupling this approach with reverse genetic techniques would potentially prove or disprove the findings of the forward genetic approach, in the identification of the gene/genes involved in conferring drug resistance.

Techniques that enable gene deletion are essential for the analysis of gene function. The approach used here is based on the PCR amplification of DNA constructs that encode a selectable marker gene (internal region) flanked by DNA sequence identical to the homologous target locus (external region). In order to generate selectable knock – out cassettes, two oligonucleotides were designed for each ORF. The first oligonucleotide has a 20 – 30 bp sequence at the 3′ end homologous to the 5′end of the selectable marker and at the 5′ end has a 40 – 80 bp sequence homologous to the target gene. The external regions therefore direct the homologous recombination at the target locus and insert the antibiotic selectable marker (Gaud *et al.*, 1997; Shen *et al.*, 2001). The major advantage of this technique is that it allows the rapid manipulation of genes without the need for cloning steps, which can be very time consuming. Using this strategy, I report the identification of a candidate gene involved in the drug resistance mechanism.

I decided to undertake an allele knock–out approach in order to determine the Cymelarsan drug resistance determinant as opposed to an RNAi based approach. The rationale for this was that as TREU 927 is heterozygous for the allele that determines resistance based on the genetic analysis (Chapter 4), it would be difficult to predict the

phenotype, if RNAi was used to knock down expression of both alleles. If the resistant allele was knocked out the prediction would be that TREU 927 would become sensitive to Cymelarsan. This strategy and the predicted outcomes are illustrated diagrammatically in Fig 6.1 A. Thus, if an allele at one of the six ORFs does determine resistance, then 50% of the knock–outs should lead to a change in Cymelarsan sensitivity. In ORFs not determining resistance, no change in phenotype would be predicted. Similarly, using an allele knock–in approach, the prediction would be that if the resistance allele of TREU 927 replaced the sensitive allele in the homozygous STIB 247 then there would be a change in phenotype i.e. to resistance (Fig 6.1 B). If, however, the sensitive allele of TREU 927 replaced the sensitive allele of STIB 247, then there would be no change in phenotype. Therefore, if any of the six ORFs were involved in conferring the resistance determinant then there should be a change in phenotype due to allele deletion or replacement.
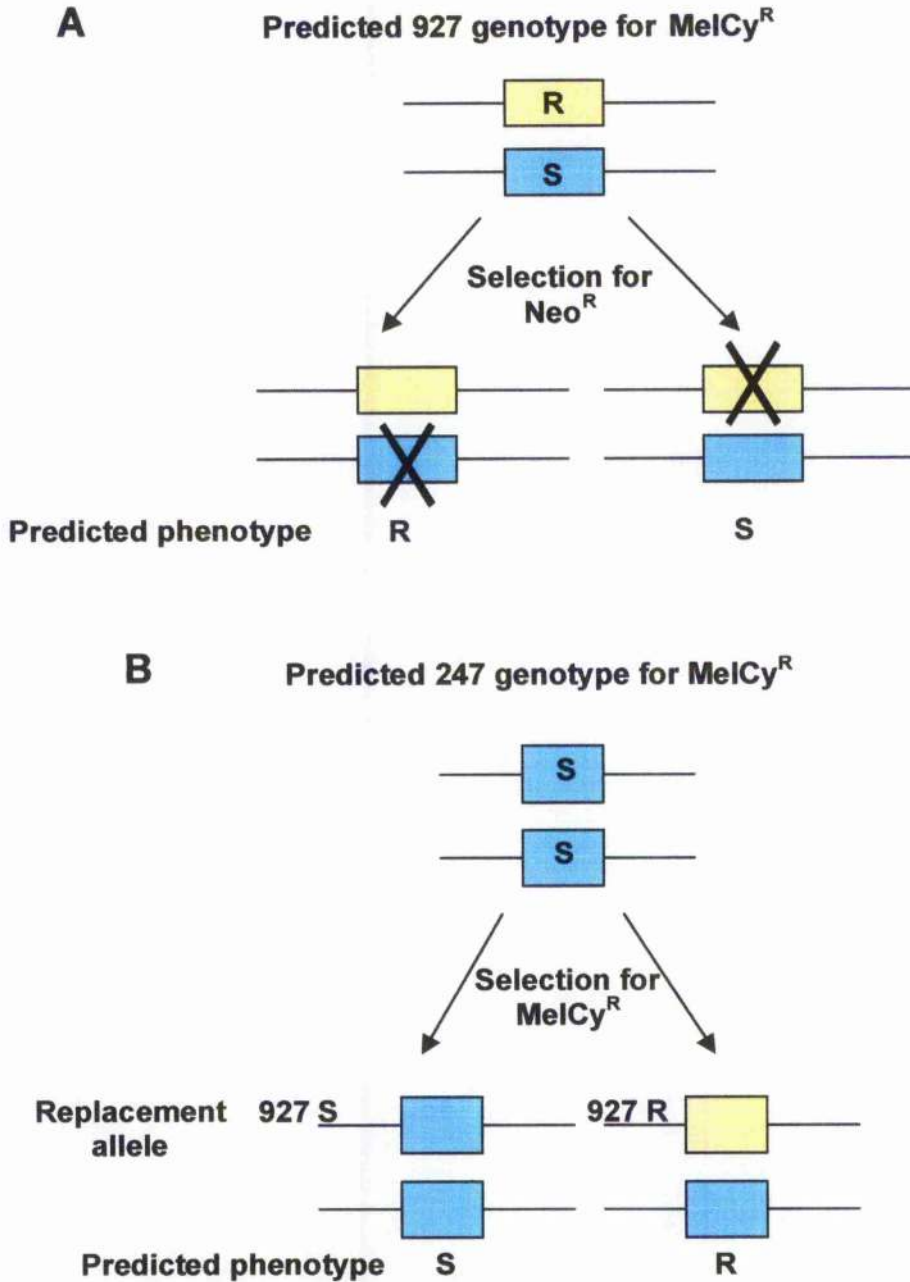
Fig 6.1: PCR based allele deletion and replacement. A: Knock–out of the resistant and sensitive allele in TREU 927. If the resistant allele (yellow) was knocked–out then the prediction would be that there would be a change in phenotype to sensitivity in the transfected cells. If, however, the sensitive allele (blue) was knocked–out then the prediction would be, no change in phenotype i.e. the transfectants would remain resistant. **B:** Allele replacement of the sensitive STIB 247 allele with the resistant TREU 927 allele would lead to the prediction that the transfectants would change phenotype to resistant. If the sensitive allele of STIB 247 was replaced with the sensitive allele of TREU 927 then the prediction would be there would be no change in phenotype.

6.2 MATERIALS AND METHODS

6.2.1 Primer design for gene deletion

Primer design was carried out according to Gaud *et al.*, (1997). The 876 bp long fragment used for disruption of the ORFs under investigation was generated by PCR using 1ng of a neomycin plasmid construct (A kind gift from Keith Matthews, University of Manchester) containing the neomycin resistance gene as a template DNA (Gaud *et al.*, 1997). The oligonucleotides used as primers (Table 6.1) in the PCR contain two distinct regions: 24bp in the internal regions were designed to amplify the selectable marker gene, and 41bp at the external regions of the primers were identical to the flanking sequences of the gene to be disrupted (Fig 6.2). PCR amplification with these primers was carried out as described in Materials and Methods, section 2.2.8 and Table 6.1. The PCR amplified deletion constructs were cleaned up and extracted using Qiagen A1Quick PCR purification kit and the DNA was recovered by ethanol precipitation. Ethanol precipitation was carried out as follows; 20µl of 3M sodium acetate plus 0.5 mls of 100% ice-cold ethanol was added to the DNA pellet and left at -20°C for 3hrs or overnight. The DNA was then ultracentifuged at 13,000 g for 15mins at 4°C. The supernatant was removed and the pellet was washed with 70% alcohol and ultracentrifuged again at 9,000 g for 5 mins. The pellet, 6 - 10µg, was then allowed to dry in a flow cabinet and re-suspended in 10µl sterile water and used directly for electroporation.

Primer	5´ - 3´ sequence
1 ORF1 - F	ccttgttaatttggttattaacatgagatgccctctgataatttaatgt tctctaagatttttatgttgacaccgtcatgattgaacaagatggattg c
2 ORF1 - R	gcaagtaagccagctggaggggggggaaacagacattattttacaatcac tttaacacatatccatggccgccgtagtgctcagaagaactcgtcaaga ag
1 ORF2 - F	gatccactaaattcacaaactccacacgtttccttacgtgtatatatga ttgaacaagatggattgc
2 ORF2 - R	ccctttaaccgccgtaaaggggggggaagaaaaataatgttttagagtcag aagaactcgtcaagaag
1 ORF3 - F	cgctcccaacaaaatatcacgattcacgtgctgtcgcattggggcgcgt attaaacgtgcgctactaccacaatgatgattgaacaagatggattgc
2 ORF3 - R	gaggcaagacaaccgtagggaacgttgcttaggaaattttcaacacgct tttcggcacgccatgtcatcgtcagaagaactcgtcaagaag
1 ORF4 - F	ccacatatctatattcatcgttacgcagctacaatccactatgattgaa caagatggattgc
2 ORF4 - R	agcgcctcaaacagtgtagccgaacccatgcgctcagaaatcagaagaa ctcgtcaagaag
1 ORF5 - F	ggagtccaccctaatgggaccacattaaatcttacctttgatgattgaa caagatggattgc
2 ORF5 - R	aaaaataacccattactttgaagatatgcattatgcctctcagaagaa ctcgtcaagaag
1 ORF6 - F	aacggcagttccctggcagttccgatgattcaccttcccgatgattgaa caagatggattgc
2 ORF6 - R	aaaataaataaataaacacacgtgtatgtgcatgtgtgtgtcagaagaa ctcgtcaagaag

Table 6.1: Oligonucleotide sequences for each ORF deletion. These primers flank the sequence of each ORF. At the end of each primer are the nucleotides homologous to the neo^R gene sequence that will direct homologous recombination of the neo^R gene in place of the ORF (indicated in red) (Fig 6.1). The PCR conditions were as follows; 95°C for 50 sec., 56°C for 50 sec. and 65°C for 50 sec. for 30 cycles. The products were separated on a 1 % Seakem agarose gel, stained with ethidium bromide and visualized by UV illumination as described in Chapter 2, Materials and Methods.
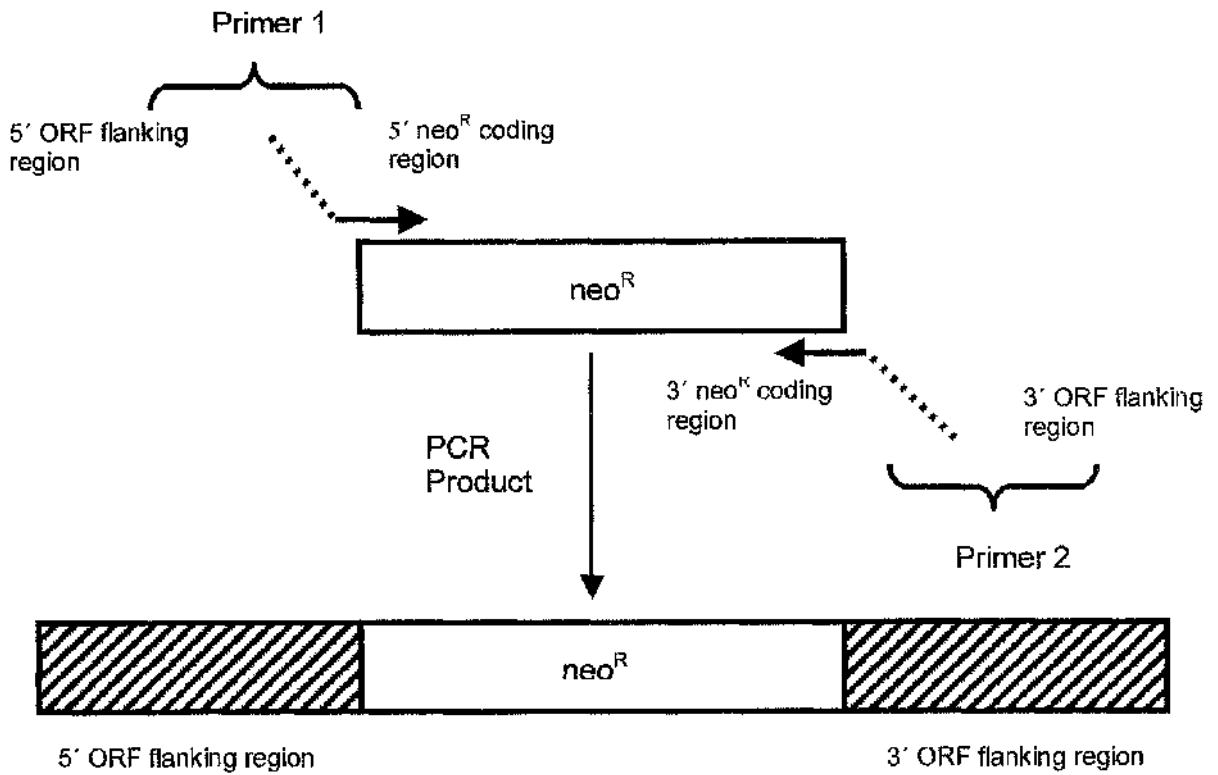
Fig 6.2: Diagrammatic representation of PCR based gene deletion technique and subsequent integration into the ORF locus. The arrows represent the position of oligonucleotides.

6.2.2 Zimmermann post fusion media (ZM buffer)

This buffer comprises of 132mM NaCl, 8mM KCl, 8mM Na_2HPO_4, 1.5mM KH_2PO_4, 0.5mM Mg acetate and 90μM $CaCl_2$, and was filter sterilized, aliquoted into 50ml falcon tubes and stored at -20°C. For electroporation of bloodstream forms 0.5g glucose / 50ml was added.

6.2.3 Transfection of procyclic form trypanosomes

Procyclic stage cells in mid log phase of growth (TREU 927) were used, at a density of approximately 5×10^6 cells/ml. Flasks were prepared with 10mls of pre-warmed SDM–79 supplemented with 30% foetal calf serum. The electroporator was set to 1,500 Volts and 25 μF. Approximately 5×10^7 were pelleted at 1,100 x g, for 7 min, for each transfection. Each pellet was resuspended in 10mls of room temperature Zimmerman post fusion media (ZM buffer) and pelleted again as above, this was repeated twice. Each pellet was then resuspended in 0.5mls ZM buffer and transferred into the tube containing the 6 - 10μg of DNA of the PCR transfection amplicon and mixed gently. This was then placed into a 0.2cm sterile electrode gap cuvette and placed in an electroporator. Cells were electroporated twice with a 10 sec gap. The electroporated cells were then immediately transferred to flasks containing the pre-warmed SDM–79 medium and incubated at 25°C. Drug selection was applied the following day. Care was taken to avoid leaving cells in the ZM buffer for any longer than necessary and cell pellets were prepared for a maximum of four transfections at any one time. The reason for this is that the ZM buffer causes cell lysis after ~ 20 min exposure (Richard Burchmore, personal communication).

6.2.4 Drug Selection of transfected procyclic forms

After 24hr recovery period geneticin (G418) (Sigma) was added to 15µg / ml. After 7 days, the dosage was increased to 100µg / ml. G418 resistant trypanosomes became apparent after 2 weeks. These cells were then centrifuged at 1,100 g for 7 mins so that the dead cells could be removed. The live cells concentrated at the bottom of the falcon tube and the dead cells were removed with the supernatant. The live cells were then placed back in SDM–79 with 30 % foetal calf serum prior to cloning.

6.2.5 Transfection of bloodstream form trypanosomes

Two ICR mice were inoculated with STIB 247 (Materials and Methods, section 2.2.2) for each transfection in order to obtain sufficient numbers of cells for transfections. The blood was differentially centrifuged at 1,100 g for 7 min. The top layer (serum and buffy coat) was decanted and centrifuged again (as above) to pellet the cells. The procedure given above for electroporation was then carried out, using the same number of cells and quantity of DNA construct. After electroporation the cells were transferred to flasks containing pre-warmed Cunninghams medium and incubated at 37°C for one hour to allow cells to recover. Immunosuppressed mice (Materials and Methods, section 2.2.2) were then inoculated with the transfected cells. After 24 hr each mouse was treated with a single dose of 1 mg/kg, 2mg/kg or 5 mg/kg Cymelarsan and their parasitaemia monitored (Materials and Methods, section 4.2.3).

6.2.6 Diagnostic PCR

DNA was extracted from the geneticin resistant trypanosomes 2–3 weeks after electroporation. A diagnostic PCR was performed in order to verify if the integration of the neomycin resistance gene had occurred at the correct position and in the correct orientation and an illustration of this is shown for one of the ORFs (Fig 6.3 and Table 6.2). The primers for these PCRs were the forward primer that flanked each of the ORFs to be deleted, but lay outside the sequence of the target gene used for the deletion construct (3) and a primer that corresponded to a sequence within the neomycin resistance gene (4) (Table 6.2).

6.2.7 Cloning of transfected cells

Procyclic clones were obtained by dilution as described in the Materials and Methods section 2.2.5. Before cloning of the transfected cells derived after knock–in, in vivo, the bloodstream forms were transformed to procyclic forms (Materials and Methods, section 2.2.4) and then cloned by dilution. Diagnostic PCR was undertaken for these clones.

6.2.8 Determination of phenotype of transfected cells

The AlamarBlue phenotype assay was conducted to determine the Cymelarsan resistance of the transfected cells as previously described in Materials and Methods section 4.2.6.

6.2.9 Sequencing of ORF 4

As ORF 4 was 2.6 kb in length it was too large to sequence with oligonucleotides flanking the ends. Therefore, eight sets of oligonucleotides were designed which, walked across the entire ORF (Table 6.3). PCR amplification of each set of primers was carried out using DNA from STIB 247, STIB 386 and TREU 927. PCR amplification of these primers was carried out as described in Materials and Methods, section 2.2.8. Conditions for amplification were: 95°C for 50secs, 56°C for 50secs and 65°C for 2mins for 30 cycles and run using 2% Seakem agarose. The PCR products were cleaned up and extracted using Qiagen A1Quick PCR purification kit and sent for sequencing to the Molecular Biology Sequencing Unit, University of Glasgow.

Primer	5´ - 3´ sequence	Product size
3 DPORF1 - F	gttacgacatgaaggtatctcccatcc	126 bp
3 DPORF2 - F	caaagaaggtgggagcgaaatcagaagg	127 bp
3 DPORF3 - F	cagtgtacgagacaaacattcccttgcggc	129 bp
3 DPORF4 - F	gtatggttaaacaatgagtgtatg	123 bp
3 DPORF5 - F	gtgacagggagcgagcagcttcaa	123 bp
3 DPORF6 - F	gctgcagccgactagtgaccgact	123 bp
4 Diagnostic primer Neo	gttgtgcccagtcatagccgaatagcctctccac	

Table 6.2: Oligonucleotide sequences for diagnostic primers. These were used to verify that the neo^R gene had integrated in the correct position and orientation (Fig 6.2). Column 3 gives the expected size of the band that would be observed if the neo^R gene had integrated in the correct position and orientation. PCR conditions for each primer set were as follows; 95°C for 50 sec., 56°C for 50 sec. and 65°C for 2 min. for 30 cycles. The products were separated on a 4 % NuSieve agarose gel, stained with ethidium bromide and visualized by UV illumination as described in Chapter 2, Materials and Methods.
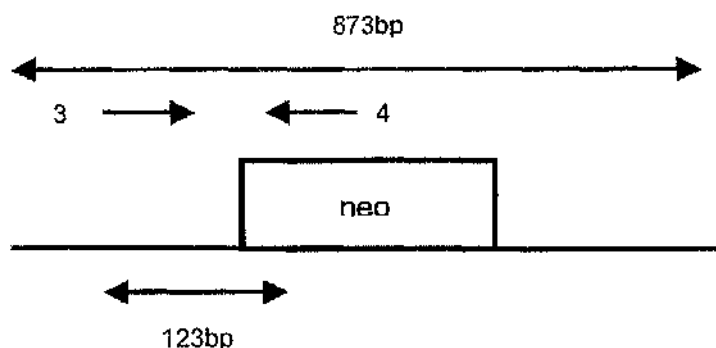
Fig 6.3: Diagrammatic representation of the position of the primers used for diagnostic PCR.
These were used to verify the deletion and to determine integration of the neo[R] gene occurred in
the correct position and orientation. The arrows represent the position of oligonucleotides used
to verify the deletion, 3 flanked ORF 4 (in this example) but lay outside the deletion construct.
Primer 4, which corresponded to a sequence within the neo[R] gene, was used in conjunction with
primer 3 to determine that the integration of the neo[R] gene occurred at the correct position (See
Table 6.2 for diagnostic primers for each ORF) (Gaud *et al.*, 1997).

Primer	5´ - 3´ sequence
WALKORF4 - AF	gccttgcgtgaccagcattggtttgc
WALKORF4 - AR	cagctgtcgtccacgcaaaaggtg
WALKORF4 - BF	gcaatcgcgcatccctcactttag
WALKORF4 - BR	gtccaggcgccgcctcacatatgt
WALKORF4 - CF	gtgcttaaccacggttgcgaaag
WALKORF4 - CR	ggaagatggcggtgaagcaacctc
WALKORF4 - DF	cagcagtttcttacgaagtggttc
WALKORF4 - DR	cagcgagatgtggtgcatcgcgc
WALKORF4 - EF	ccacgttggtcatcacggaattg
WALKORF4 - ER	cgactacgagcatgctggttacag
WALKORF4 - FF	cgccgcgatacagggcgagcgttg
WALKORF4 - FR	cgccactggttcgtatgagaagg
WALKORF4 - GF	gacgagttgtgacctccgcgcccac
WALKORF4 - GR	cctgcatcagtagcggcagctgga
WALKORF4 - HF	gttgaacttaggaagctgcgtttc
WALKORF4 - HR	gttctaggatctgcgcgcgcatag

Table 6.3: Oligonucleotide sequences used for primer walking of ORF 4.

6.3 RESULTS

6.3.1 Allele knock-outs

The primers used to generate the targeting constructs for ORFs 1 – 6 consisted of sequence complementary to the neomycin resistance (neo^R) gene flanked to the 5′ side by >40 bp from the 5′ untranslated region (UTR) and to the 3′ side by >40 bp from the 3′ UTR of each of the six ORFs. The length of the DNA sequence primer required for either a high efficiency of transfection or a high rate of integration is dependent on the size of the gene which is being disrupted so therefore the primers varied in size from 40 – 80 bp (Shen *et al.*, 2001), the oligonucleotides used for this study are listed in Table 6.1. The UTR sequences were chosen so that recombination between the neo^R gene and the flanking sequences occurred at the initiation and stop codons of the targeted gene. As a result the translation of the selectable marker depended solely on the regulatory sequences flanking each of the target genes (Fig 6.2).

T. brucei line TREU 927 was cultured as procyclic forms in SDM-79 with 10% foetal calf serum and electroporated with 10μg of linear DNA containing the neo^R gene and the flanking homologous sequence to each of the six ORFs. The trypanosomes were put under drug selection 24hr after electroporation using geneticin (G418) and left for two weeks (See Materials and Methods, section 6.2.4). DNA was extracted from the geneticin resistant trypanosomes and the diagnostic PCR was performed in order to determine whether integration of the neo^R gene had occurred (Fig 6.3).

A diagnostic PCR was carried out using primers 3 and 4 that corresponded to a sequence within the neo^R gene (Table 6.2). A 123 – 129 bp product would be expected

for each of the ORFs (Table 6.2) if the integration of the neo^R gene occurred in the correct position and this was observed (Fig 6.4).
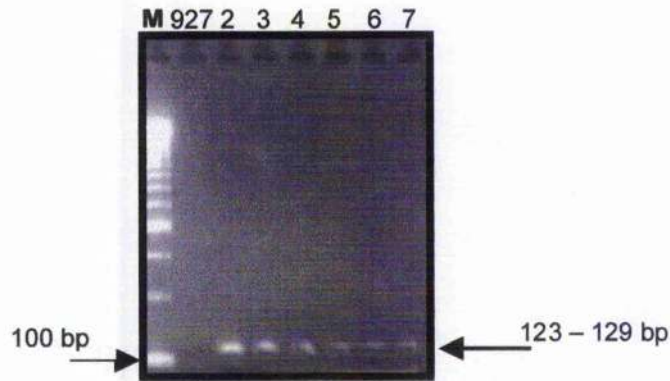
Fig 6.4: Diagnostic PCR for transfections of TREU 927 cells with neo^R using primers 3 and 4. In lane 1, a negative control using non-electroporated DNA from the parental line TREU 927. Lanes 2 – 7 were the cells electroporated with neo^R and selected with G418 and represent each of the six ORFs respectively. This showed that the 123 bp predicted fragment was present and confirmed that the neo^R gene had integrated at the correct position. **M:** 100 bp DNA ladder (Invitrogen Life Technologies).

The next step of the analysis was to phenotype the uncloned transfected TREU 927 cells which had been selected for knock–out of a single allele of each of the six ORFs i.e. a change to sensitivity in the presence of increasing concentrations of Cymelarsan. The drug sensitivity assay, AlamarBlue, was used to test this hypothesis. The results obtained indicated that one ORF (number 4) now had an intermediate resistance phenotype (Fig 6.5). It should be stressed that the assay was conducted in the population of cells before cloning. Two explanations for an intermediate level of resistance can be considered. Firstly, as the transfected cells had only been under selection in G 418 for seven days, it was likely they contained both transfected and wild type cells. Secondly, as only one allele of ORF 4 will have been deleted, half the

transfectants would be predicted to be resistant to Cymelarsan. To further characterise

the transfected population with respect to these factors, clones were established.
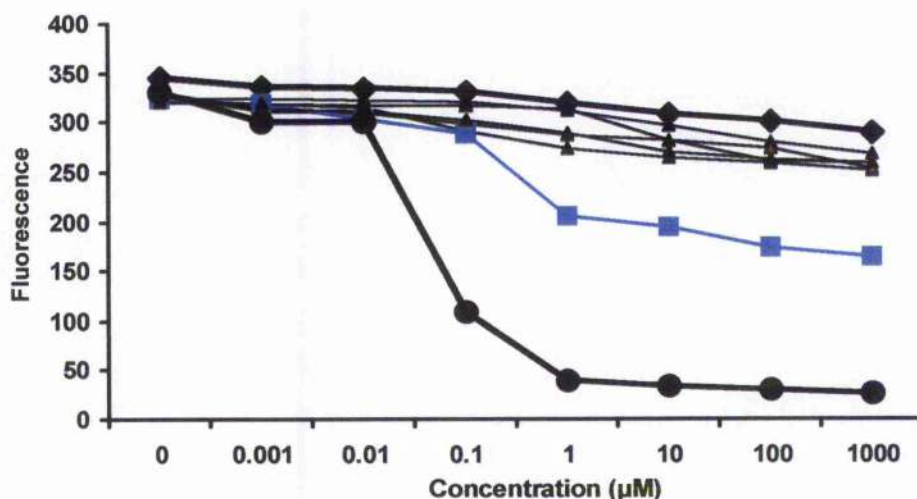
Fig 6.5: AlamarBlue drug sensitivity assay with Cymelarsan after deletion of alleles of each of the six ORFs. The analysis indicated that there was an effect on the resistance phenotype for ORF 4 (■) that demonstrated an intermediate phenotype in comparison to the resistant line TREU 927 (◆) and the sensitive line STIB 247 (●). The other five ORFs (▲) all followed the same resistance pattern as TREU 927 suggesting they were not involved in conferring the resistance phenotype. The x-axis represents the measure of fluorescence at OD_{590}, and the y-axis represents the increasing concentration of Cymelarsan.

After cloning, the cells transfected for ORF 4 these were tested, by PCR, to determine

which contained the neo^R gene. Six of 16 clones were found to contain the neomycin

insert (Fig 6.6). These were then assayed to determine if their phenotype had changed

from resistant to sensitive, using the AlamarBlue screening assay.
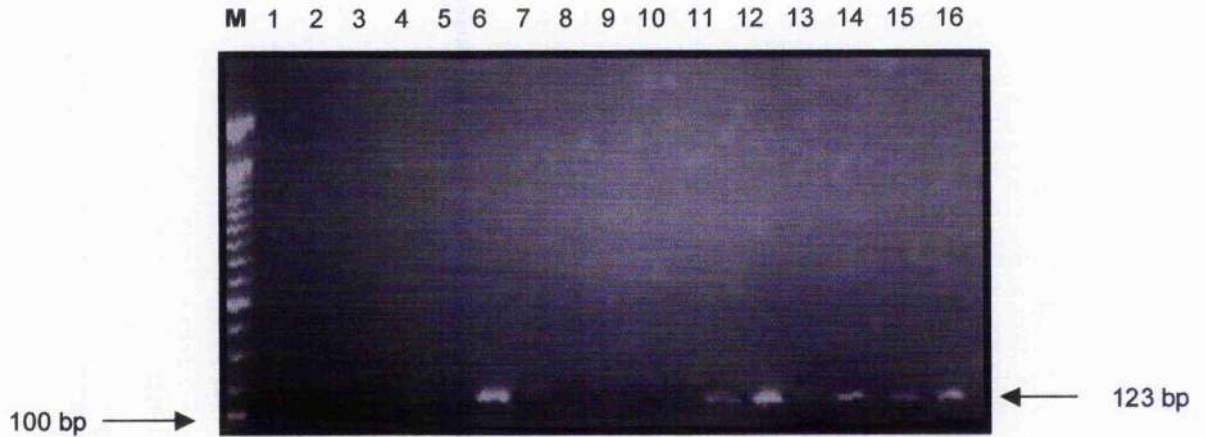
Fig 6.6: Diagnostic PCR to determine presence of neomycin insert in procyclic clones derived from transfection of ORF 4. Of all the clones tested for the neomycin insert only six were found to contain the insert in the correct position and orientation 6, 11, 12, 14, 15 and 16 above respectively. A 123 bp product would be expected if the integration of the neo[R] gene occurred at the correct position, and this was observed.

Phenotypic analysis showed that four of the six neo[R] containing transfected clones conferred a change to a sensitive phenotype with IC_{50} values in the range of $1 - 5$ μM (Fig 6.7) which were typically $85 - 90\%$ of those observed with the non-transformed STIB 247 sensitive line (Chapter 4). It was also found that one of the clones had a intermediate phenotype, the simplest explanation for this is that this was not a clone and was a mixture of resistant and sensitive cells, but this was not formally tested.
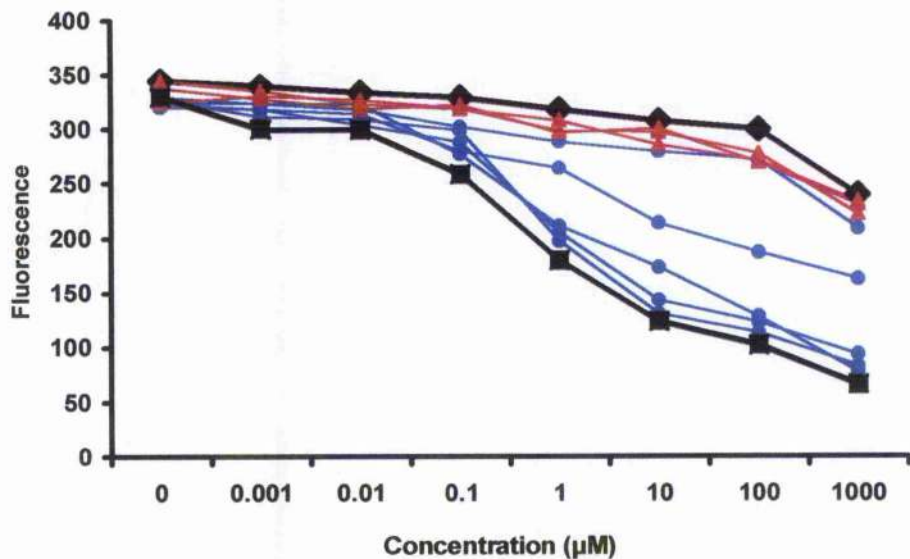
Fig 6.7: Phenotype analysis of the six procyclic clones derived after replacement of ORF 4 with neo^R gene. Four of the clones gave a sensitive phenotype when tested with increasing concentration of cymelarsan, one remained resistant and one gave an intermediate phenotype (●). The four clones represented by (▲) are clones that after PCR analysis did not contain the neo^R gene and retained the resistant phenotype as would be expected. Wild type parental lines represented by: TREU 927 (◆), STIB 247 (■). The x-axis represents the measure of fluorescence at OD_{590}, and the y-axis represents the increasing concentration of Cymelarsan.

To confirm and extend the finding that disruption of one allele of ORF 4 in TREU 927 resulted in a sensitive phenotype and therefore that one allele of TREU 927 ORF 4 determined the Cymelarsan resistance phenotype, knock–in experiments were designed. The plan was to transfect the sensitive line STIB 247 with both alleles of TREU 927 ORF 4 and then select for Cymelarsan resistance. If one of the alleles of TREU 927 ORF 4 determines resistance and is integrated into STIB 247, the resulting transfectants should be resistant to Cymelarsan.

Firstly, I determined if ORF 4 was present in line STIB 247, using the same oligonucleotides used to amplify ORF 4 in TREU 927, WALKORF 4 AF and HR, (Table 6.3), were used. After PCR amplification a product the same size as that obtained for ORF 4 in TREU 927 was observed, thus confirming that ORF 4 was present in STIB 247 (Fig 6.8).
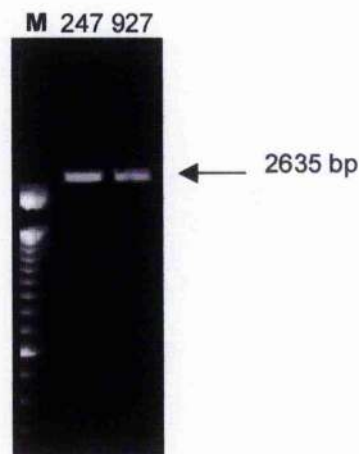
Fig 6.8: PCR analysis to determine presence of ORF 4 in STIB 247. Oligonucleotides designed to amplify ORF 4 in TREU 927 were used to determine if ORF 4 was present in line STIB 247. As can be seen from the gel, a band of the same size was observed in STIB 247 as in TREU 927, used as a positive control.

6.3.2 Allele knock–in

Transfection of ORF 4 in STIB 247 was then conducted, replacing one allele with an equivalent allele from TREU 927 using the same primers as those used for the knock–out but without the neo sequence at the end (Table 6.1). After one round of transfection, eight immunosuppressed mice were inoculated with the transfected cells. After 24hr four of the mice were treated with 1 mg/kg Cymelarsan and four with 2 mg/kg Cymelarsan and their parasitaemia monitored. After four days five of the eight mice developed parasitaemia, three treated with 1 mg/kg and two treated with 2 mg/kg

Cymelarsan. Cells, therefore, capable of surviving at the same drug concentrations as the wild type resistant line TREU 927 (>2 mg/kg) over the same time period as reported previously (Chapter 4) were produced. The remaining three inoculated mice did not produce an infection and therefore may have required another round of transfection.

The transfected bloodstream form lines were transformed to procyclic forms and then cloned by limiting dilution. Five clones were established in procyclic culture and were then analysed for their drug resistance phenotype (Fig 6.9). Four of the clones showed a Cymelarsan resistance profile equivalent to that of the parental stock TREU 927 while one gave a sensitive phenotype. These data, therefore, indicated that there was a clear discrimination between resistance and sensitivity responses in cell lines that have either had ORF 4 deleted, or replaced with ORF 4 from a Cymelarsan resistant line.
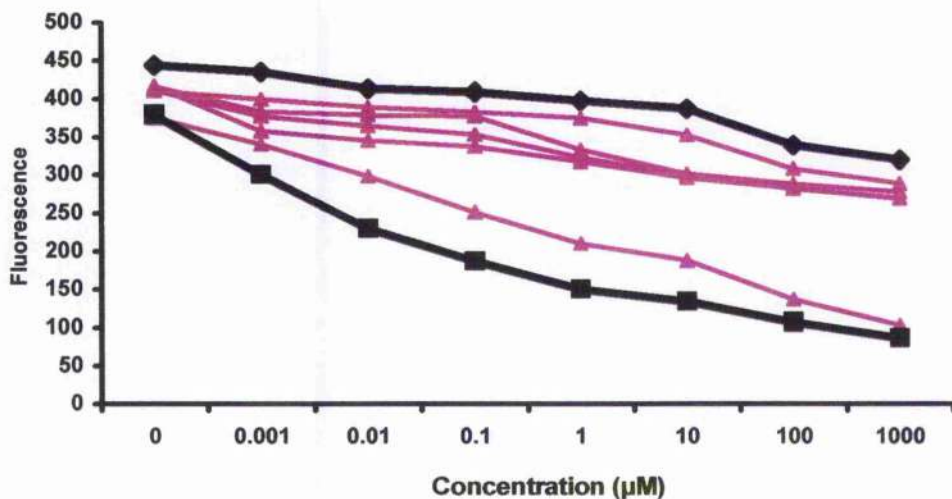
Fig 6.9: **Phenotypic analysis of procyclic clones derived after transfection of ORF 4 TREU 927 into bloodstream forms of STIB 247.** Four of the five cell lines tested (▲) showed a resistance phenotype similar to that of the resistant wild type TREU 927 line (◆). One of the transfected cell lines followed the pattern similar to the wild type sensitive line STIB 247 (■) which may have been due to insufficiency of selection. The x-axis represents the measure of fluorescence at OD_{590}, and the y-axis represents the increasing concentration of Cymelarsan.

6.3.3 Cross–Resistance

As a result of the transfection experiments, two sets of clones were available. One set had one allele of ORF4 deleted and were sensitive to Cymelarsan while the second set had the resistant allele of ORF4 from TREU 927 in the STIB 247 sensitive stock and were Cymelarsan resistant. By testing the resistance/sensitivity phenotype of these clones to the arsenicals, Melarsoprol, Trimelarsan, Melarsen oxide and Phenylarsine Oxide and the diamidines, Berenil and Pentamidine, it was possible to determine whether ORF4 was also the determinant of resistance to these compounds. Drug assays revealed that the six clones that had ORF 4 replaced with the neo^R gene in TREU 927 four, that were sensitive to Cymelarsan, were also sensitive to all the other drugs tested except Pentamidine (Fig 6.10 A-F). Similarly, the five procyclic clones derived after ORF 4 in STIB 247 was replaced with alleles of ORF 4 from TREU 927, and were resistant to Cymelarsan also became resistant to all the drugs tested except Pentamidine (Fig 6.11).

Fig 6.10 A – F: AlamarBlue drug sensitivity assay with the four clones that had ORF 4 replaced with the neo^R gene in TREU 927. (A) Melarsoprol, (B) Trimelarsan, (C) Melarsan oxide, (D) Phenylarsine oxide, (E) Berenil, and (F) Pentamidine. STIB 247 (■), TREU 927 (◆).
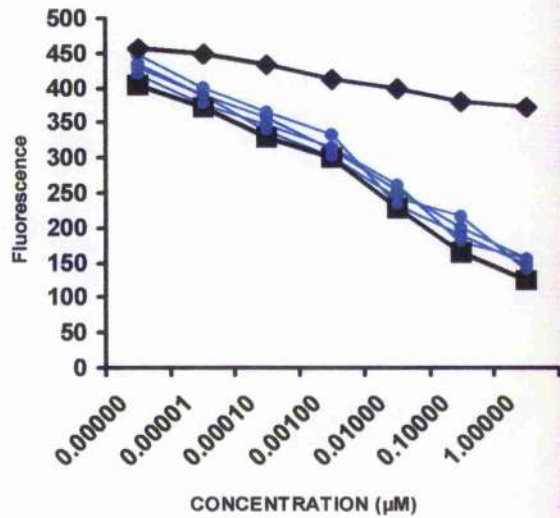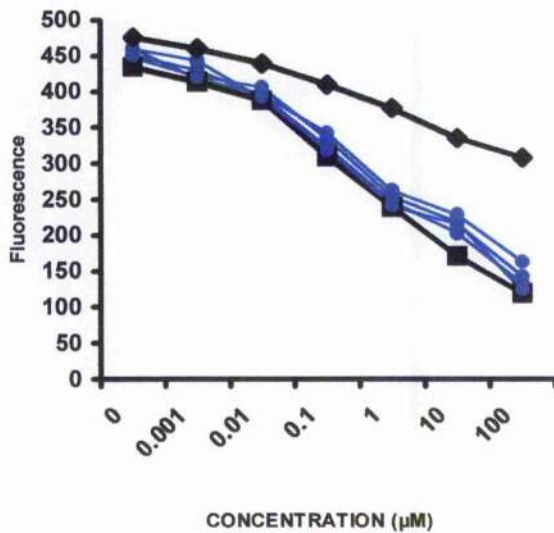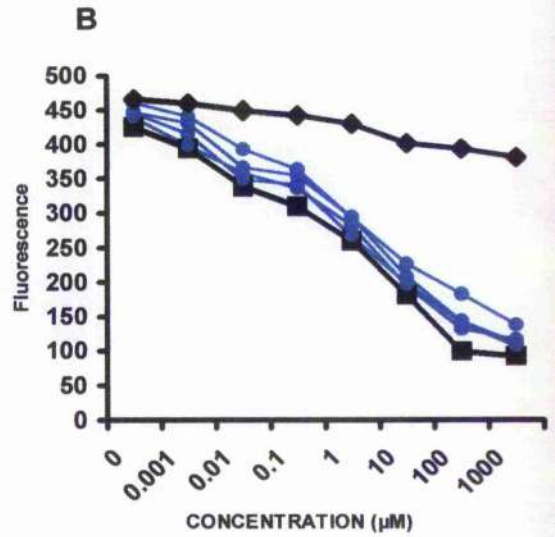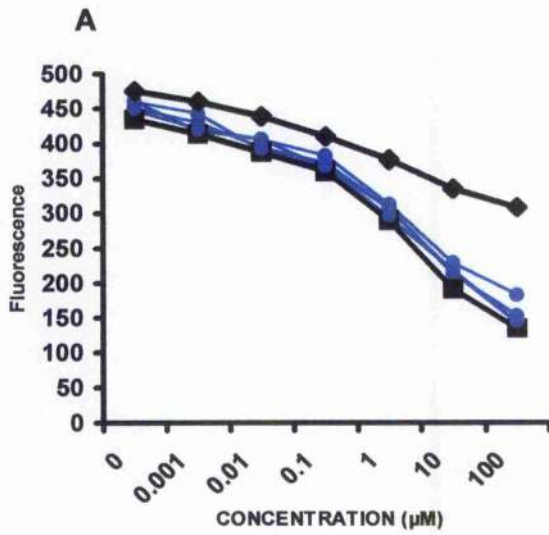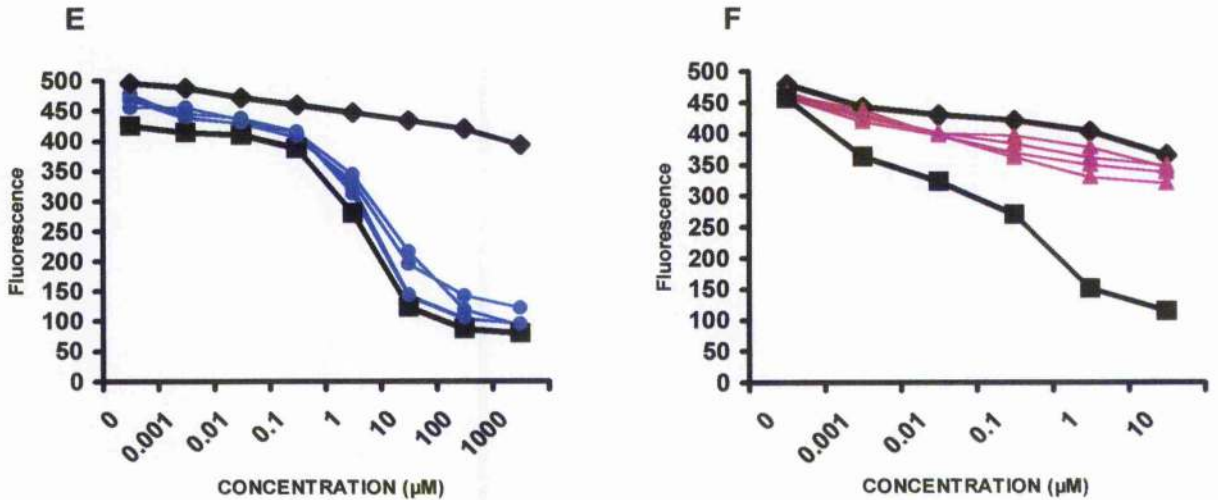
Fig 6.10 :A – F represent Melarsoprol, Trimelarsan, Melarsan oxide, Phenylarsine oxide, Berenil, and Pentamidine, respectively. After replacement of ORF 4 with the neo^R gene in TREU 927, it was found that four clones derived after transfections and found to have changed phenotype from resistant to sensitive for Cymelarsan were also sensitive to Melarsoprol, Trimelarsan, Melarsan oxide, Phenylarsine oxide and Berenil. This suggested that the deletion of ORF 4 changed the phenotype of these four clones for these drugs as well as Cymelarsan, therefore the same cross resistance pattern observed in Chapter 4 between Cymelarsan and these five drugs was observed here. However, when these clones were assayed using Pentamidine, there was no change in phenotype, they remained resistant (F). Therefore, it is clear that the resistance conferred by Pentamidine must be due to another gene, which was thought to be the case after the results found in Chapter 4. STIB 247 (■), TREU 927 (♦), Sensitive F1 progeny (●), Resistant F1 progeny (▲). The x-axis represents the measure of fluorescence at OD$_{590}$, and the y-axis represents the increasing concentration of each drug.

Fig 6.11 A – F: AlamarBlue drug sensitivity assay with the four clones that had ORF 4 in STIB 247 replaced with ORF 4 from TREU 927. (A) Melarsoprol, (B) Trimelarsan, (C) Melarsan oxide, (D) Phenylarsine oxide, (E) Berenil, and (F) Pentamidine. STIB 247 (■), TREU 927 (◆).
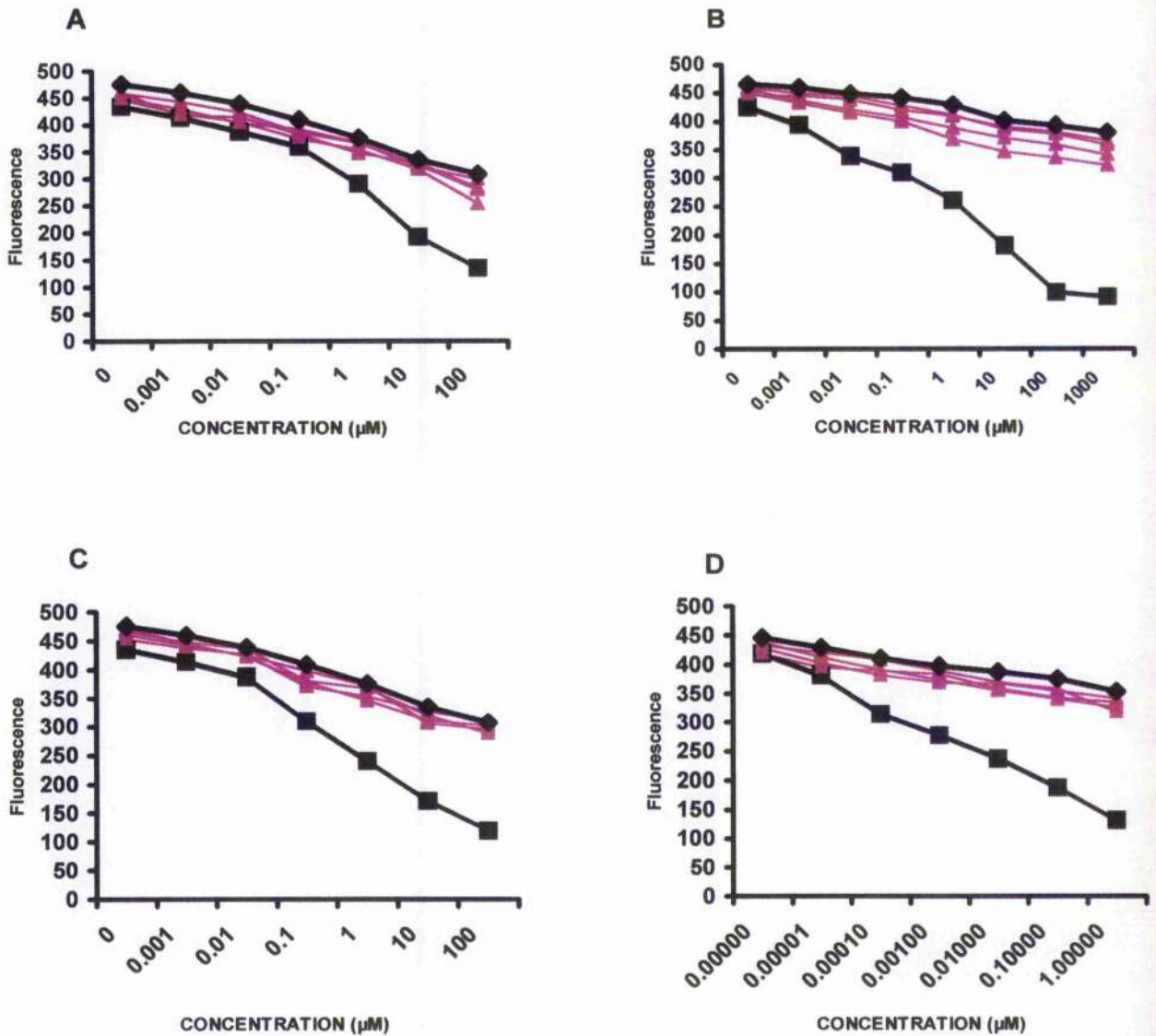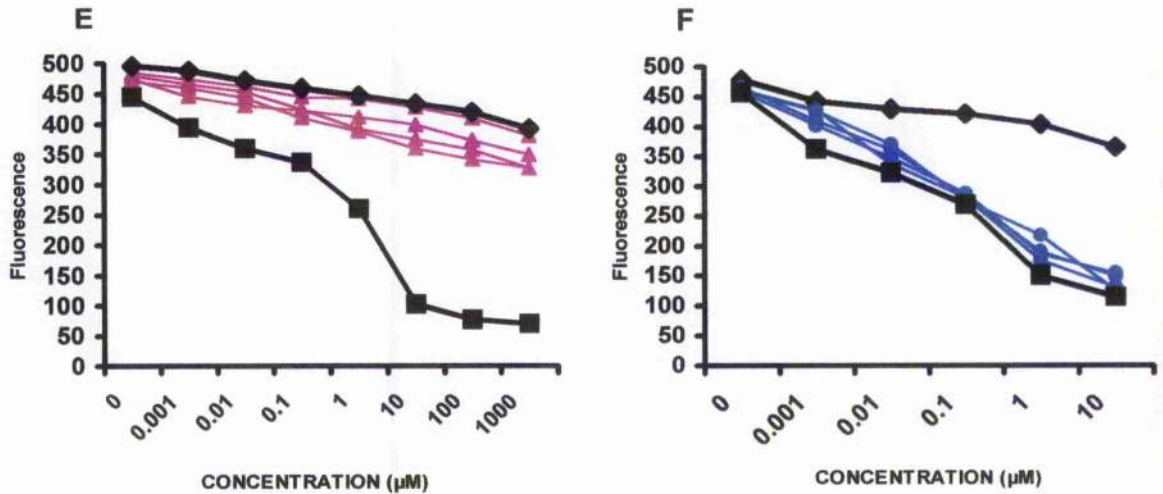
Fig 6.11: A – F represent Melarsoprol, Trimelarsan, Melarsan oxide, Phenylarsine oxide, Berenil, and Pentamidine, respectively. After replacement of ORF 4 in STIB 247 with ORF 4 from TREU 927, it was found that four clones derived after transfections and found to have changed phenotype from sensitive to resistant for Cymelarsan were also resistant to Melarsoprol, Trimelarsan, Melarsan oxide, Phenylarsine oxide and Berenil. This suggested that the replacement of ORF 4 changed the phenotype of these four clones for these drugs as well as Cymelarsan, therefore the same cross resistance pattern observed in Chapter 4 between Cymelarsan and these five drugs was observed here. However, when these clones were assayed using Pentamidine, there was no change in phenotype, they remained sensitive (**F**). Therefore, it is clear that the resistance conferred by Pentamidine must be due to another gene. STIB 247 (■), TREU 927 (◆), Sensitive F1 progeny (●), Resistant F1 progeny (▲). The x-axis represents the measure of fluorescence at OD_{590}, and the y-axis represents the increasing concentration of each drug.

Taken together, these data demonstrate that allelic differences in the coding sequence of ORF 4 determine resistance to Cymelarsan, and cross–resistance to Melarsoprol, Melarsen oxide, Trimelarsan, Phenylarsine oxide and Berenil. The results obtained from AlamarBlue drug sensitivity assay for Pentamidine after allele deletion and replacement of ORF4, however, did not follow this pattern (Fig 6.10 and 6.11 F). The results found in Chapter 4, where all the 38 F1 progeny from the STIB 247 x TREU 927 cross were sensitive to Pentamidine, suggested that resistance to Pentamidine was conferred by a

different gene to that determining Cymelarsan resistance. The results obtained from allelic deletion and replacement of ORF 4 and subsequent drug assays of the transfected cells with Pentamidine show that lack of segregation of Pentamidine resistance is not due to the dominance relationships at the same locus, but establish that another gene must be responsible.

ORF4 is formally named Tb927.2.2380 (http://www.genedb.org) but is also termed 25N14.75 (El-Sayed et al., 2003). Translation of the 2.6 kb TREU 927 ORF4 DNA sequence predicted an 874 amino acid, 96 kDa protein with an isoelectric point (pI) value of 8.72, which is a 'conserved hypothetical protein' or 'T. brucei family hypothetical protein' (El-Sayed et al., 2003). PIX (Protein Identifier X) analysis (www.hgmp.mrc.ac.uk), predicted that there were 4 transmembrane (Tm) domains within ORF 4. Of these four domains, however, only one was thought to be a true Tm domain as it gave a prediction score of 'excellent'. The preferred model of orientation of this domain, which was predicted by PIX, was from the inside to outside (Fig 6.12). This Tm domain is situated close to the N–terminus and starts at amino acid 12 and ends at amino acid 32. It may function to anchor the protein in a membrane; however, as this is a small Tm domain, spanning only 21 amino acids it is thought unlikely to be a transporter. Also, using PIX, it was found that there were no Pfam or prosite hits. Pfam is a large collection of multiple sequence alignments covering many common protein domains and families. Prosite determines if there are any post–translational modifications in common with other protein families. As analysES with these programmes were negative, it suggested that ORF 4, a hypothetical protein, has not

been characterised in any other organism to date in the protein databases nor does it contain any common functional domains.
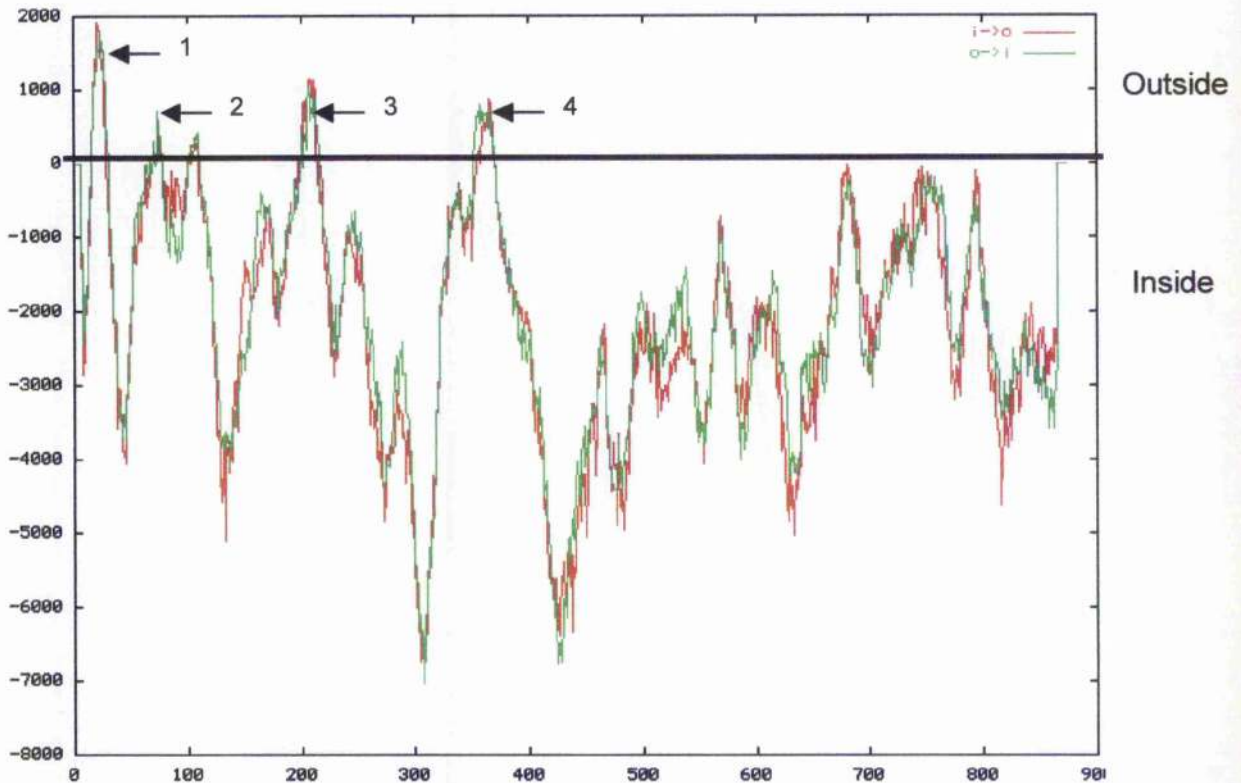
Fig 6.12: Indicating four predicted transmembrane (Tm) domains for ORF4 using PIX analysis. Of these four Tm domains only one was a true Tm domain with the quality of the prediction being 'excellent' (1), whilst the others were either 'good' or 'poor' (2 - 4). The preferred model of orientation of the Tm domain 1 is from inside to outside (red on graph) which starts at position 12 and ends at position 32 of the 874 amino acid protein sequence. The position of this domain in ORF4 protein sequence is shown in Fig 6.12.

Database blast searches, using the DNA sequence of ORF 4, were carried out to determine any sequence homology to the other chromosomes of *T. brucei*. This revealed that there was some homology to a contig on Chromosome IX, giving 100%

homology over a 371 bp region. There was also some homology to a *T. cruzi* clone, giving sequence identity of 65% over 234 bp and to *Leishmania major* chromosome II where there was 66% sequence homology over 105 bp. Searches of sequence homology to *Toxoplasma gondii, Plasmodium falciparum, P. vivax, P. chabaudi, Eimeria tenella, Theileria annulata* and the yeasts *Schizosaccharomyces pombe, Candida albicans* and *Saccharomyces cerevisiae* revealed no significant homology of any of these organisms with ORF 4 from *T. brucei.* Blast searches were also conducted against the human, rat and mouse genomes but again there was no sequence homology with ORF 4.

The forward and reverse genetics data show that ORF4 is heterozygous in TREU 927 for an allele that determines resistance to Cymelarsan. In order to further test this conclusion and screen for polymorphisms that might determine resistance, the entire 2.6 kb region of ORF 4 for lines STIB 247 and TREU 927 was amplified by PCR and the products sequenced to detect any polymorphisims in ORF 4 between STIB 247 and TREU 927. Due to the fact that ORF 4 was 2.6 kb in length oligonucleotides were designed to produce PCR products spanning the length of ORF 4 (Table 6.3).

Molecular Biology Sequencing Unit (MBSU), Glasgow University, carried out the DNA sequencing of STIB 247 and TREU 927 and comparisons were made using the ABI Prism software (www. Technelysium.com.au). A single base difference was found when the two sequences were aligned, a change from a T in TREU 927 to a C in STIB 247 that was homozygous in both stocks. It would be predicted that TREU 927 would be heterozygous for a base substitution leading to an amino acid change in order to

account for the genetic analysis of Cymelarsan resistance. On examination, however, of the chromatograms derived from sequencing STIB 247 and TREU 927 ORF 4 revealed that the base change in TREU 927 was not heterozygous i.e. there was no overlapping of this peak with another peak representing a different base (Fig 6.13). This base change, therefore, could not be responsible for conferring the resistance phenotype.

TREU 927
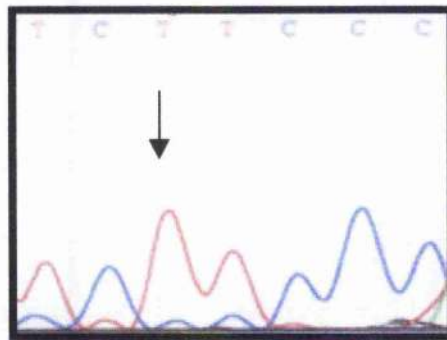
STIB 247
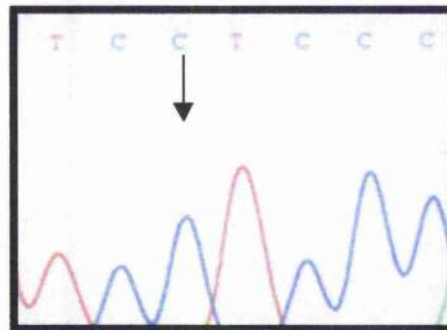
Fig 6.13: Chromatograms showing peak profiles generated for of STIB 247 and TREU 927. As can be observed the polymorphism detected from sequencing ORF 4 in TREU 927 was not heterozygous, it was quite clearly homozygous as there was no overlapping with another peak (base) (Fig 6.14 A – C) and therefore, could not be used as an informative polymorphic marker to determine the Cymelarsan resistance phenotype for a population of cells.

By examining the chromatograms, by eye, it was found that there were seven positions in the sequence of TREU 927 where the base calling was ambiguous and TREU 927 could be potentially heterozygous (Fig 6.14 A – C). This was due to the fact that the

peaks were overlapping and so discrimination between two bases was difficult. For example in Fig 6.14 A 1 – 3, there were three potential heterozygosities in TREU 927, where instead of being either (1) C or T, (2) A or T and (3) G or T, these bases were called the bases found at these points in the STIB 247 sequence i.e. C, A and T, respectively. There are other points at which overlapping occurs in the figures below but these were not thought to be true heterozygosities as the overlap was not very pronounced e.g. in Fig 6.14 A2 (red arrow) the overlap where G could be T is not very pronounced and so I have not considered it as a possible polymorphism.

Fig 6.14 A – C : Chromatograms showing peak profiles generated for STIB 247 and TREU 927.
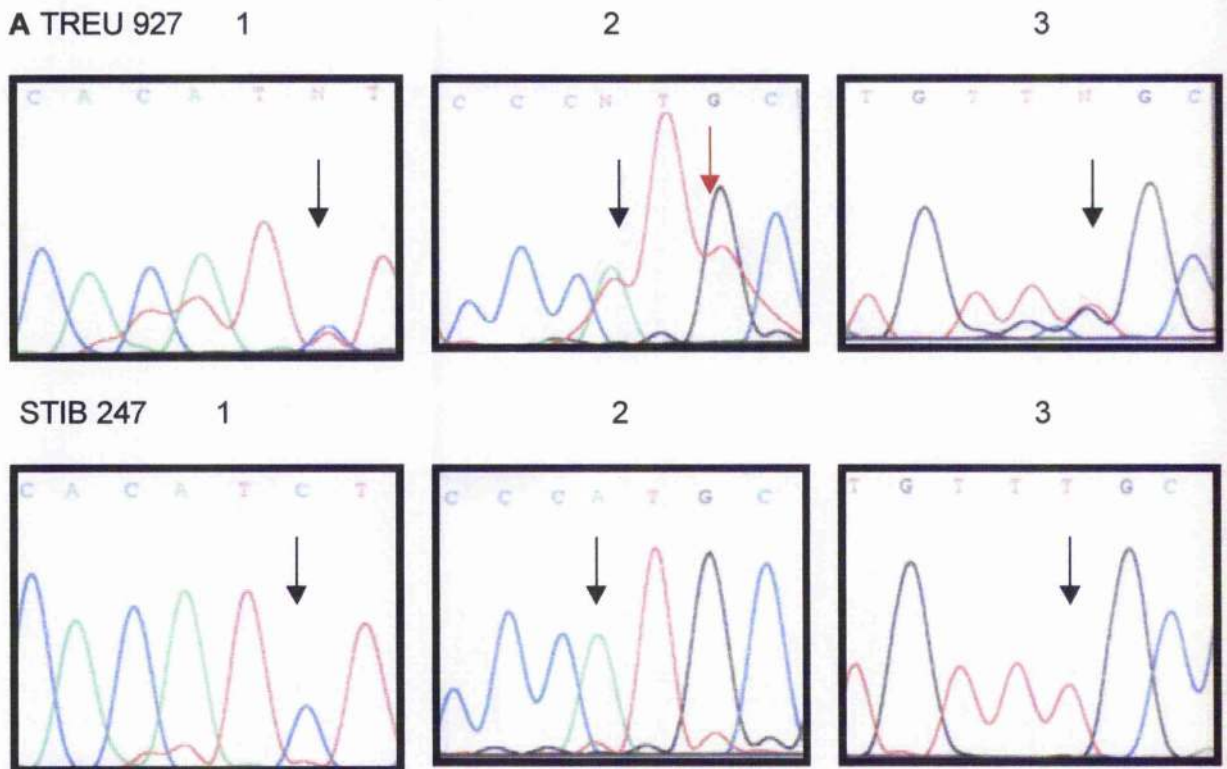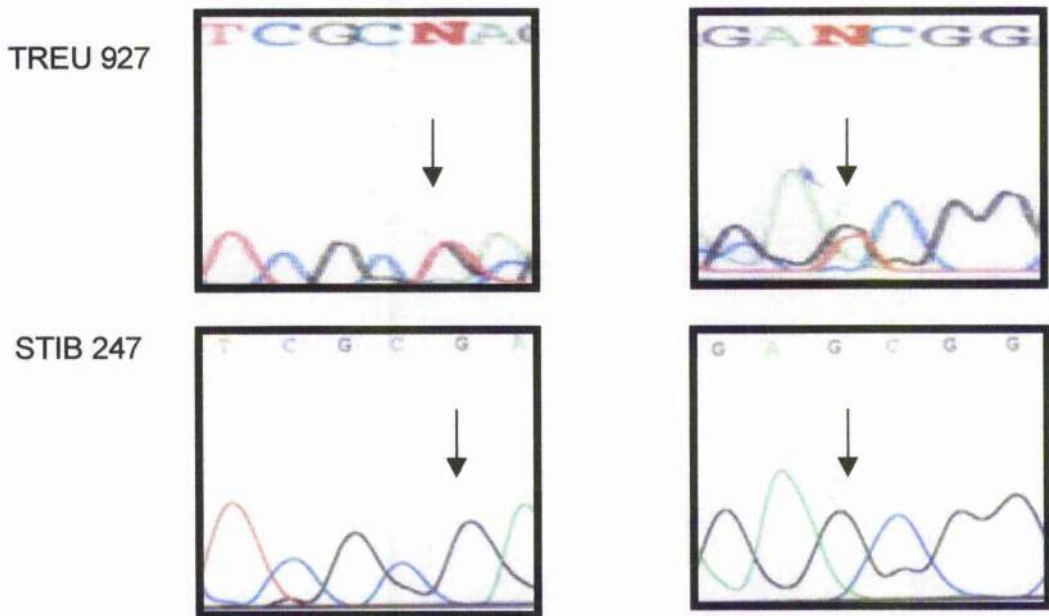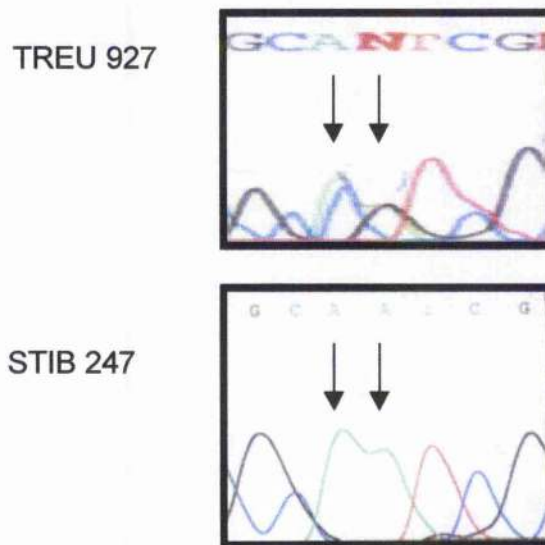
A) Interpretation of peaks for which TREU 927 could be heterozygous. Due to the fact that the peaks indicated (arrow) were overlapping at these points the base could not be defined and were represented by N. Chromatogram of the equivalent regions for STIB 247 where the peaks in 1, 2 and 3 are clearly distinguishable in comparison to the same peaks in 1, 2 and 3 of TREU 927. The remaining four possible regions where TREU 927 could be heterozygous due to overlapping bases are shown in **(B)** and **(C)** with the corresponding region in STIB 247 where no overlap was observed.

These putative polymorphisms where TREU 927 was heterozygous (Fig 6.15) could, however, be attributed to; 1) poor sequencing; 2) the sequences were obtained by direct sequencing from a PCR product using Taq polymerase and as there was no proof reading activity, these could be 'jackpot' mutations. On this basis, the sequencing should be repeated using a mix of Taq and pfu (proof reading) polymerases (15:1) (Krawchuk and Wahls, 1999). Alternatively, the PCR product could be cloned and multiple clones sequenced to define the two alleles from stock TREU 927.

```
CTACAATCCACTGCACACTTCTCCACAGTGGTACCATTTCCAAGGGCTTTTGGCATTCTT
TTGCACACAGCCGAAATCATCTTCTTGAGTGCATATCATTCTGCCACTGCGACTTGAGCG
CAACCCAACATGCTGAAGAAACGACTCGCG/TAGAG/TCGGCGGGATCCGAGTCCATATTTCTC
CACGTGTTTAAGCGCCGCTTCCCCGTCACTTCTCAACCCCCGCGCAGCGTTGGATTGGCC
AAATAGCACAAAGTTCAAGCGCACCAAACCCCGCA/CA/GTCGCGCATCCCTCACTTTAGAGGA
CATGTTTGCGACTTTGTTATTACCGCTTGTTCCATTCCCGGCACCTTTTGCGTGGACGAC
AGCTGCAAAAGCAATGGGCTCAGTCGGGGCGTAAAAATCCCAGCCGTGCGTCCACAACCG
CCCCGCAAGTAAAACGGCCTCTTCATCTGGTGTCAGGAAACCCATGTACGGGTCCAGTGG
AACGTGATTCCCAGCATTGCCACTGCTACTATTATTGTGGCTATTTTGGTGGTCTGCTGC
GTCATGCGAGAAATCAAAGAAGGCACCTGCGCGAGTGAAAAGGAAATCTGTGCTTAACCA
CGGTTGCGAAAGTGACGAAGGAAAGCTTTCCTGTCGCCTGCTTCCAAGGCACACCGCATC
ATTCTTCTCATTACACCAATGGATGGGTAGAGGCTCCATATACCGCCGATCGCGCAGGAC
ATATGTGAGGCGGCGCCTGGACTCATATCTCAATTTGAATTTTGCTCCCTCTCCCCCCGA
CACAGTGGCGCTAACGTCCTCCCCATTTTCCACGTTGCCACAACCACTGCTGCCGCCGTC
GCTTTTCGCATCGCACACTTTCCGGCAACCTTCTTCGCTGTGCTGCATCTTTACTGCCAC
ACCACATACGTAGGAGGTGGTGTTTTGTTCACTCATACTTGTCAGCAGTTTCTTACGAAG
TGGTTCACGCAATTTCTTCATCCAATACAATTGGGCACTATTTGGCATCCGGGTTCTCAC
ATTTGGGATCGAGGCTCCGTTTCGCGGGTAGTTGAGAGGTTCTGCTCCAGAGGTTGCTTC
ACCGCCATCTTCCTCTGTGGGAAGTCGGAAAAGGAACTCCACAACATCTTCACTCCCGAA
TCCTCTGGCGCCTTCCGCAGCACTTTCGCCGTTTCCATCCCCCTTCTCTTTGTCCTCCAT
TCCATCGGAACTGTGTAAACGCAGTCCTTCAAGATCGTACAGAGCAGATCCACTCCTTTT
CTTTGAAGGATTTCTCTCGTGAGCTACGTTGAAGTAATCTTCCGTCCCTCCCCTTTCCTT
TCCACGTTGGTCATCACGGAATTGTTGATCCCCATGATCCTTAACCCCTTCATGTCTGTG
ACCTTCATTGCTTACGTGGCGGACCACAACTTCTTCCCCA/TTGCGCGATGCACCACATC/TTCG
CTGTATGATGTTT/GGCCATCTGCGAGAGTATAGGCCGTCGTGACGGGAGCCTTAGTAGCAT
GAGCCGCGCTGTCACATCCCATGAGGGCACTAACAACACGCCTGCATTAAGAAACATCAC
GTATGTTTCGCCGCGATACAGGGCGAGCGTTGCATACCGCTGGGAGGCAATGGAGGTAAA
GTCACTGAGCCCTCCACTTGCATCACTCCCACTTGAAGCATCCCCATCCCTGTAACCAGC
ATGCTCGTAGTCGTCCTCAGCTCTTCGACTGCGTGGATTTTCTTTTTTTTCACTCACCTT
GACGTTACCTTCGCCTCCACCCATATTGCTTCCTTTGCTGTGCCTTCGTGACTTAGCCGA
CACACGCAGTTGCCGCAGCCGTATGTTGTCCCGTGGACAGAATCCATACCGTTCGGCGCA
CGTGTGAGACTGATAAGCAACAGGAATACATGGAGACGATGGTGGGAAAGGCCACCGGGA
CGAGTTGTGACCTCCGCGCCCACCCGATTCCTCAGCCGTCATTACGTCGGGGAGGAACTC
AACGATACCGGTGAAAATAGCAACTGACCAAAGTGCCGATTCATATAAGTTGTGAACTGT
CATAGCACACACACTCTCTAGAGAAACAGACCTTCTCATACGAACCAGTGGCGTTTCATG
GTGCAGAAGACCAACGAAGATACTACCCTCGAGCCCGTAGCCGCGGCGTGCGACATGAGT
TGGACTCCAGTACGTGTCGGAATGTGTGACATTCTTTATGGAGCCATTGTCCAACCTCTT
TTCTAACTCATTGTTGAACTTAGGAAGCTGCGTTTCATAGTATTCCAGCAAAATGGCCGC
GGGCGTTGCATTCGGGGGCAAACCAAACATATCCGTGGCTCCTCCAGCTGCCGCTACTGA
TGCAGGCATTCCATCAGTTTCCTGGTCCACGAATGCCGTCAATACTGACAGAAGTTTCGT
GGTAAATGAAAAAACCGCTGGCGAGGCCCGCAAGTTTCCGACCCCTGGCAAACCACCCCG
AACAGCATGGCTTCCTTCACCAATGTGGCCACCTCTCATTCTGTTTTTACGCTCGCGCGA
CATATTATTCCATAACAAGAAGGTAACCATGACTAAGGCTGGGAGCACCATATACATCGG
GACGGTTATGCAACGCCGGCCCCGACGCCGTGTTTCTGAGCGCAT
```

Fig 6.15: **Sequence of ORF 4 3´ - 5´ (El-Sayed *et al.*, 2003).** The seven polymorphisms that are putatively heterozygous for TREU 927 are shown. The bases that are lightly shaded are those, which appear in the sequence of ORF 4. Represented in red, blue and black (bold) are the alternative bases if TREU 927 was heterozygous at these points. The base in TREU 927 that was found to different from STIB 247 but was homozygous for TREU 927 (Fig 6.13) is also shown (darkly shaded).

The protein sequences of STIB 247 and TREU 927 were obtained using Jemboss Transeq software available through HGMP (www.hgmp.mrc.ac.uk) and aligned using Multalin (http://npsa-pbil.ibcp.fr/cgi-bin/align_multalin.pl). Firstly, the bases in the TREU 927 sequence for the seven regions at which TREU 927 was putatively heterozygous were changed (Fig 6.15). For example in Fig 6.14 A1, in the sequence of ORF 4 (El-Sayed et al., 2003) the position N was defined as C but could be a T, so this was changed to T and the same was undertaken for the other six polymorphisms in Fig 6.14 A – C. Comparisons revealed four codon differences (two base changes were next to each other and were covered by one amino acid change; Fig 6.15) after the seven base changes had been made, between the drug sensitive line STIB 247 and the drug resistant line TREU 927 at positions (amino acid numbering) ^{Arg} 49 ^{Ser}, ^{Leu} 91 ^{Arg} (two bases in one codon), ^{Trp} 472 ^{Arg} and ^{Asp} 478 ^{Asn}, (Fig 6.16). A summary of the results is shown in Table 6.4.

```
927                                                                          LGS
247                                                                          LGS
Consensus                                                                    LGS
Prim.cons                                                                    LGS
```

49

```
927        CVEGCHYXKWPSKANKQVCGFDDEQTCIMRGSRSSRLGVHQLFSESSRRSGLGYKEVHKL
247        CVEGCHYXKWPSKANKQVCGFDDEQTCIMRGSRSSRLGVHQLFSERSRRSGLGYKEVHKL
Consensus  CVEGCHYXKWPSKANKQVCGFDDEQTCIMRGSRSSRLGVHQLFSErSRRSGLGYKEVHKL
Prim.cons  CVEGCHYXKWPSKANKQVCGFDDEQTCIMRGSRSSRLGVHQLFSE2SRRSGLGYKEVHKL
```

91

```
927        AAEGDSRLGRAANSQGFLVFNLRVLGRRRADRVKSSMNAVKNNGSTGNGAGKAHVVAAFA
247        AAEGDSRLGRAANSQGFLVFNLRVLGRLRADRVKSSMNAVKNNGSTGNGAGKAHVVAAFA
Consensus  AAEGDSRLGRAANSQGFLVFNLRVLGRrRADRVKSSMNAVKNNGSTGNGAGKAHVVAAFA
Prim.cons  AAEGDSRLGRAANSQGFLVFNLRVLGR2RADRVKSSMNAVKNNGSTGNGAGKAHVVAAFA
```

```
927        IPETPAYFDWGHTWLRGALLVAEEDPTLFGMYPDLPVHNGANGSSSNNHSNQHDAADHSF
247        IPETPAYFDWGHTWLRGALLVAEEDPTLFGMYPDLPVHNGANGSSSNNHSNQHDAADHSF
Consensus  IPETPAYFDWGHTWLRGALLVAEEDPTLFGMYPDLPVHNGANGSSSNNHSNQHDAADHSF
Prim.cons  IPETPAYFDWGHTWLRGALLVAEEDPTLFGMYPDLPVHNGANGSSSNNHSNQHDAADHSF
```

```
927        DFFAGARTFLFDTSLWPQSLSSPFSEQRRSGLCVADNKENCWHIPLPEMYRRDRLVYTLR
247        DFFAGARTFLFDTSLWPQSLSSPFSEQRRSGLCVADNKENCWHIPLPEMYRRDRLVYTLR
Consensus  DFFAGARTFLFDTSLWPQSLSSPFSEQRRSGLCVADNKENCWHIPLPEMYRRDRLVYTLR
Prim.cons  DFFAGARTFLFDTSLWPQSLSSPFSEQRRSGLCVADNKENCWHIPLPEMYRRDRLVYTLR
```

```
927        RRSEYRLKFKAGEGGSVTASVDEGNEVNGCGSSGGDSKADCVKRCGEESHQMKVAVGCVY
247        RRSEYRLKFKAGEGGSVTASVDEGNEVNGCGSSGGDSKADCVKRCGEESHQMKVAVGCVY
Consensus  RRSEYRLKFKAGEGGSVTASVDEGNEVNGCGSSGGDSKADCVKRCGEESHQMKVAVGCVY
Prim.cons  RRSEYRLKFKAGEGGSVTASVDEGNEVNGCGSSGGDSKADCVKRCGEESHQMKVAVGCVY
```

```
927        STTNQESMSTLLKKRLPERLKKMWYLQASNPMRTRVNPISAGNRPYNLPEAGSTAEGGDE
247        STTNQESMSTLLKKRLPERLKKMWYLQASNPMRTRVNPISAGNRPYNLPEAGSTAEGGDE
Consensus  STTNQESMSTLLKKRLPERLKKMWYLQASNPMRTRVNPISAGNRPYNLPEAGSTAEGGDE
Prim.cons  STTNQESMSTLLKKRLPERLKKMWYLQASNPMRTRVNPISAGNRPYNLPEAGSTAEGGDE
```

```
927        ETPLRFLFEVVDESGFGRAGEAASEGNGDGKEKDEMGDSSHLRLGELDYLASGSRKKSPN
247        ETPLRFLFEVVDESGFGRAGEAASEGNGDGKEKDEMGDSSHLRLGELDYLASGSRKKSPN
Consensus  ETPLRFLFEVVDESGFGRAGEAASEGNGDGKEKDEMGDSSHLRLGELDYLASGSRKKSPN
Prim.cons  ETPLRFLFEVVDESGFGRAGEAASEGNGDGKEKDEMGDSSHLRLGELDYLASGSRKKSPN
```

472   478

```
927        REHAVNFYDETGGREKGRQDDRFQQDGHDKVGEHRHGENSVHRVVVEERARHVVNRQIIN
247        REHAVNFYDETGGREKGRQDDRFQQDGHDKVGEHRHGENSVHRVVVEEWARHVVDRQIIN
Consensus  REHAVNFYDETGGREKGRQDDRFQQDGHDKVGEHRHGENSVHRVVVEErARHVV#RQIIN
Prim.cons  REHAVNFYDETGGREKGRQDDRFQQDGHDKVGEHRHGENSVHRVVVEE2ARHVV2RQIIN
```

```
927        AMQSLIPRRSPLRLLMLRATVDWSPVLLVGANLFMVYTEGRYLALTAYRQSAISTFDSLG
247        AMQSLIPRRSPLRLLMLRATVDWSPVLLVGANLFMVYTEGRYLALTAYRQSAISTFDSLG
Consensus  AMQSLIPRRSPLRLLMLRATVDWSPVLLVGANLFMVYTEGRYLALTAYRQSAISTFDSLG
Prim.cons  AMQSLIPRRSPLRLLMLRATVDWSPVLLVGANLFMVYTEGRYLALTAYRQSAISTFDSLG


927        GSADSGSSADGDRYGAHEYDDEARRSRPNEKKESVKVNGEGGGMNSGKSHRRSKASVRLQ
247        GSADSGSSADGDRYGAHEYDDEARRSRPNEKKESVKVNGEGGGMNSGKSHRRSKASVRLQ
Consensus  GSADSGSSADGDRYGAHEYDDEARRSRPNEKKESVKVNGEGGGMNSGKSHRRSKASVRLQ
Prim.cons  GSADSGSSADGDRYGAHEYDDEARRSRPNEKKESVKVNGEGGGMNSGKSHRRSKASVRLQ


927        RLRINDRPCFGYREACTHSQYAVPICPSSPPFPWRSSNHGGRGGSEEATMVDPLFEVIGT
247        RLRINDRPCFGYREACTHSQYAVPICPSSPPFPWRSSNHGGRGGSEEATMVDPLFEVIGT
Consensus  RLRINDRPCFGYREACTHSQYAVPICPSSPPFPWRSSNHGGRGGSEEATMVDPLFEVIGT
Prim.cons  RLRINDRPCFGYREACTHSQYAVPICPSSPPFPWRSSNHGGRGGSEEATMVDPLFEVIGT


927        FIAVSWLASEYLNHVTMACVSELSVSRRMRVLPTEHHLLGVFISGELGYGRRAVHTPSWY
247        FIAVSWLASEYLNHVTMACVSELSVSRRMRVLPTEHHLLGVFISGELGYGRRAVHTPSWY
Consensus  FIAVSWLASEYLNHVTMACVSELSVSRRMRVLPTEHHLLGVFISGELGYGRRAVHTPSWY
Prim.cons  FIAVSWLASEYLNHVTMACVSELSVSRRMRVLPTEHHLLGVFISGELGYGRRAVHTPSWY


927        TDSHTVNKISGNDLRKELENNFKPLQTEYYELLIAAPTANPPLGFMDTAGGAAAVSAPMG
247        TDSHTVNKISGNDLRKELENNFKPLQTEYYELLIAAPTANPPLGFMDTAGGAAAVSAPMG
Consensus  TDSHTVNKISGNDLRKELENNFKPLQTEYYELLIAAPTANPPLGFMDTAGGAAAVSAPMG
Prim.cons  TDSHTVNKISGNDLRKELENNFKPLQTEYYELLIAAPTANPPLGFMDTAGGAAAVSAPMG


927        DTEQDVFATLVSLLKTTFSFVAPSARLNGVGPLGGRVAHSGEGIHGGRMRNKRERSMNNW
247        DTEQDVFATLVSLLKTTFSFVAPSARLNGVGPLGGRVAHSGEGIHGGRMRNKRERSMNNW
Consensus  DTEQDVFATLVSLLKTTFSFVAPSARLNGVGPLGGRVAHSGEGIHGGRMRNKRERSMNNW
Prim.cons  DTEQDVFATLVSLLKTTFSFVAPSARLNGVGPLGGRVAHSGEGIHGGRMRNKRERSMNNW


927        LLFTVMVLAPLVMYMPVTICRRGRRRTESRM
247        LLFTVMVLAPLVMYMPVTICRRGRRRTESRM
Consensus  LLFTVMVLAPLVMYMPVTICRRGRRRTESRM
Prim.cons  LLFTVMVLAPLVMYMPVTICRRGRRRTESRM
```

Fig 6.16: Protein sequence alignment (3´- 5´) of STIB 247 and TREU 927 for ORF 4. There were four missense mutations between the two lines at amino acid positions [Arg] 49 [Ser], [Leu] 91 [Arg], [Trp] 472 [Arg] and [Asp] 478 [Asn] (**D** – Aspartic acid, **N** – Asparagine, **W** – Tryptophan, **R** – Arginine, **L** – Leucine, **S** – Serine) These mutations were located in the middle and end of the sequence away from the Tm domain. The Tm domain is highlighted in yellow. The position of the silent mutations are also indicated (shaded).

Nucleotide Sequence (3' – 5')	Region on ORF 4 where polymorphisms are located					
Base pair position	**150**	**154**	**175 and 176**	**1419**	**1437**	**1452**
TREU 927 Allele 1	CGCGAG	GAGCGG	CGCAAT	CCCATG	CATCTC	TTTGCC
TREU 927 Allele2	CGCTAG	GATCGG	CGCCGT	CCCTTG	CATTTC	TTGGCC
STIB 247	CGCGAG	GAGCGG	CGCAAT	CCCATG	CATCTC	TTTGCC

Amino Acid Sequence (3' – 5')						
Codon Position	**49**	**51**	**91**	**472**	**478**	**484**
TREU 927 Allele 1	ESS	SRR	RRR	ERA	VNR	NAM
TREU 927 Allele 2	ERS	SRR	RLR	EWA	VDR	NAM
STIB 247	ERS	SRR	RLR	EWA	VDR	NAM
Consensus sequence	ErS	SRR	RrR	ErA	V#R	NAM
Comments	1	2	3A	3B	3B	2

Table 6.4: A summary of the seven putative polymorphisims determined after sequence comparisons between TREU 927 and STIB 247 for ORF 4. Indicated are the nucleotide polymorphisms between the two lines and their position within ORF 4. Also indicated are the amino acid differences from the protein sequence of ORF 4 between the two lines. **1:** Only one of the five polymorphisms had a restriction enzyme site at codon position ^{Arg} 49 ^{Ser}. There is a restriction enzyme site with the endonuclease Bfa I in TREU 927 and Hpy188 III in STIB 247. **2:** There are two silent mutations at codon positions ^{Arg} 51 ^{Arg} and ^{Ala} 484 ^{Ala}. **3A:** For the polymorphisms at positions 175 and 176 bp one amino acid substitution covered both these polymorphisms at codon ^{Leu} 91 ^{Arg} but there were no restriction enzyme sites. **3B:** There were no restriction enzyme sites for the two polymorphisms at codons ^{Trp} 472 ^{Arg} and ^{Asp} 478 ^{Asn}. None of the amino acid substitutions caused a frame shift. **A** – Alanine, **D** – Aspartic acid, **N** – Asparagine, **W** – Tryptophan, **R** – Arginine, **L** – Leucine, **S** – Serine

6.4 DISCUSSION

The analysis of the progeny of a genetic cross between a drug sensitive line (STIB 247) and a drug resistant line (TREU 927) provided a genetic approach to investigate the basis for resistance, which showed that a locus determining drug sensitivity was located to a 25 kb segment on *T. brucei* chromosome II, a segment that contained six ORFs (Chapter 5). The model of inheritance predicts that TREU 927 is heterozygous and resistance is dominant (Chapter 4). It follows, therefore, that if the resistance allele amongst the six ORFs was disrupted (knocked-out) TREU 927 would become sensitive to Cymelarsan. As there were a relatively small number of genes within this locus it was feasible to conduct a PCR-based allelic deletion strategy to determine if any of these genes had an effect on conferring the drug resistance phenotype. Database blast searches of these six ORFs revealed no sequence homology to genes in any organisms for which sequence was available.

Phenotypic analysis of cells derived after single allele deletion of each ORF in the resistant line TREU 927 revealed that there was a significant change in the phenotype of populations of cells that had ORF 4 replaced with the neo^R gene, but this was not observed with any of the other five ORFs (Fig 6.5). Six clones from the ORF 4 transfected cell line were generated and the presence of the neo^R allele confirmed (Fig 6.6). Four of these transfectants were sensitive to Cymelarsan. One of the clones, however, remained resistant. This could have been attributed to the neo^R replacing the sensitive allele of TREU 927 ORF 4. The clone that gave an intermediate resistant phenotype could have arisen as a result of having more than one trypanosome in the well at cloning and as such there was a mix of cells containing the insert and cells that

198

did not, thus conferring an intermediate phenotype. Those clones that did not have the neo insert remained resistant, at the same level as that observed for the naturally resistant line (Fig 6.7).

To further investigate this finding, a reverse 'knock-in' strategy was employed, but selecting for resistant transfectants in a drug sensitive (STIB 247) population using Cymelarsan to select *in vivo*. After selection, procyclic forms of the transfected cells were grown and cloned. It was found, using the AlamarBlue screening assay, that four of the five clones obtained had changed phenotype from sensitive to resistant in the presence of Cymelarsan (Fig 6.9).

With the set of clones obtained from allele deletion and replacement analysis, it was possible to test if the same results were observed for the other six drugs previously used in this study. Drug assays showed that the four transfected clones containing the neo^R gene in place of ORF 4 in the resistant line TREU 927 now had a sensitive phenotype to the drugs Melarsoprol, Trimelarsan, Melarsan oxide, Phenylarsine oxide and Berenil. For Pentamidine, however, the clones did not change phenotype, which suggested that ORF 4 was not responsible for conferring the Pentamidine resistance phenotype (Fig 6.10). The four clones obtained as a result of allele replacement of ORF 4 in STIB 247 with ORF 4 from TREU 927 were all also tested with the same six drugs. It was found that for all the drugs except Pentamidine, which remained sensitive, the clones had changed phenotype from sensitive to resistant (Fig 6.11).

As a result of these findings, proof was obtained that ORF 4 conferred the drug resistance phenotype. In order to identify the polymorphisms in this gene that could be responsible for the phenotype, sequencing of ORF4 from STIB 247 and TREU 927 was carried out. PCR amplification and direct sequencing of ORF 4 initially identified one polymorphism between the two lines. On examination of the chromatogram for this polymorphism, however, it was found that TREU 927 was homozygous and therefore this polymorphism could not be responsible for the resistant phenotype (Fig 6.13). However, there were seven candidate polymorphisms found by examining the chromatograms derived from the sequencing of ORF 4 in STIB 247 and TREU 927 that were potentially heterozygous for TREU 927 (Fig 6.14).

Oligonucleotide primers could, therefore, be designed to regions in the sequence flanking these polymorphisms (Fig 6.15). It would then be possible to genotype the sensitive and resistant hybrid F1 progeny of the STIB 247 x TREU 927 cross using these primers and therefore determine which of the TREU 927 alleles was responsible for conferring resistance. The prediction is that the sensitive progeny would have inherited one allele from TREU 927 whilst the resistant progeny would have inherited the other allele. By simple PCR analysis, therefore, these oligonucleotides would be able to detect drug resistant parasites within a population of cells from an infected host. Due to time constraints this has not been achieved in this study.

There were four missense mutations in the amino acid sequence (Fig 6.16) found after substituting the bases in the ORF 4 sequence where TREU 927 could be heterozygous (Fig 6.15). One of these mutations had a restriction site, therefore PCR – RFLP analysis

could be carried out on the drug resistant and sensitive lines and any differences in banding patterns scored (For example see, Fig 5.2). At amino acid position 49 there was a restriction enzyme site for the endonuclease Bfa I in TREU 927 and Hpy188 III in STIB 247. By using either of these restriction endonucleases and conducting PCR – RFLP amplification of TREU 927, STIB 247 and the hybrid progeny from the STIB 247 x TREU 927 cross, the presence or absence of a band would be observed between the Cymelarsan resistant and sensitive lines and could be scored if this mutation was involved in conferring the drug resistance phenotype.

The codon differences at positions 91, 472 and 478, did nor alter restriction sites in either STIB 247 or TREU 927. Therefore, oligonucleotide primers would have to be designed flanking these mutations and PCR amplification of the parental and hybrid progeny in order to determine if any of these were involved in conferring the drug resistance phenotype. The remaining two single nucleotide polymorphisms (SNPs), at codon positions 51 and 484, did not change the amino acid sequence and were therefore synonymous (silent) mutations. However, this does not imply that these synonymous mutations are non – functional. A recent study has shown that some synonymous mutations in the human dopamine receptor D2 (DRD2) altered the predicted mRNA folding and led to a decrease in mRNA stability and translation. Most of the mRNA stability elements are generally located in the 5′ and 3′ UTRs of genes, however a synonymous mutation in the coding region of DRD2 affected mRNA stability (Duan et al., 2003). In order to determine, therefore, if the three synonymous mutations in this study are functional, in vitro translation of ORF 4 mRNAs carrying these mutations would have to be investigated to ascertain if any have an effect on gene

expression by comparing the amount of protein synthesis between the wild type and mutant cells (Duan *et al.*, 2003).

The PCR – RFLP technique has been used in *T. gondii* gene *GRA1*, to distinguish different strains of *T. gondii* based on the presence of an A versus a G at a single position which changes a Nla III site to a Mae III site and so it was possible to type the allele at this locus based on this single nucleotide substitution (Sibley *et al.*, 2002). This may be a useful technique to use in future studies to distinguish between alleles of sensitive and resistant strains, both laboratory derived lines and more importantly as a rapid diagnostic tool for determining resistance in field isolates. Unfortunately, time did not allow for analysis of these candidate polymorphic markers for Cymelarsan resistance.

These data suggest that ORF 4 has a significant role in conferring drug resistance in *T. brucei*. However, this does not rule out the possibility that there are other mechanisms potentially involved in conferring drug resistance, for example transporters and drug efflux mechanisms. However, the P2 transporter is unlikely to be involved in conferring the Cymelarsan drug resistance in TREU 927 for two reasons which have been identified in this study: (1) ORF 4 is located on chromosome II but the P2 transporter is on chromosome XI and there is no sequence homology between the two and (2) the results show that there was cross resistance between Cymelarsan and Phenylarsine oxide and it has been reported that Phenylarsine oxide does not enter the cell through the P2 transporter (Carter and Fairlamb, 1993). The roles of different potential mechanisms of drug resistance identified in laboratory studies need to be validated by

analysing field isolates of defined resistant phenotypes. The study reported here analyses a 'natural' resistant phenotype that was not generated by laboratory selection.

CHAPTER 7

DISCUSSION

The aim of this project was to identify the gene/genes involved in conferring resistance to the arsenical group of drugs in *T. brucei* using a classical genetic approach (starting with the phenotype and finishing with the identification of the gene). Genetic crosses were conducted between the three stocks STIB 247, STIB 386 and TREU 927, which were field isolates from Tanzania, The Ivory Coast and Kenya, respectively, and showed naturally occurring variation in their susceptibility to Cymelarsan.

Prior to the start of the project crosses had been made between the three stocks in all pair-wise combinations and a total of 37 progeny clones isolated that, by marker analysis, corresponded to the F1 progeny produced by mating between each pair of parental stocks. In order to undertake the genetic analysis of phenotype using these crosses, it was necessary to increase the number of independent F1 progeny isolated from these crosses in order to statistically test different models of inheritance and undertake linkage analysis. A further 42 independent progeny clones were isolated.

The analysis involved four phases aimed at identifying the drug resistance determinant. Firstly, the determination of the inheritance of the resistant phenotype leading to a genetic model of inheritance, secondly, linkage analysis for each chromosome, thirdly, fine scale mapping of a candidate locus and finally screening of the candidate genes using a PCR–based allele knock–out and knock–in strategy. Discussion of the results obtained in these four phases has been provided at the end of each of the results

chapters. Therefore, here the main conclusions from this study are discussed, the implications in relation to future work considered and the relevance of the results to drug resistance in the field.

From the segregation of the resistant phenotype in the F1 progeny, it was postulated that resistance is determined by two alleles at a single locus where resistance is heterozygous and dominant (Chapter 4). With complete genetic maps available for all eleven *T. brucei* chromosomes (MacLeod *et al*, in preparation), linkage analysis and fine scale mapping were undertaken to localise the resistance determinant to a 25 kb region on chromosome II containing six ORFs (Chapter 5). The final part of this study was to use a reverse genetic approach to determine if any of the six ORFs were involved in conferring the drug resistance trait. This was achieved by using a PCR based allele deletion approach in the resistant parental stock. If any of the ORFs contained an allele that determined resistance then a change in phenotype would be observed when this allele was replaced with the neomycin selectable cassette. Only allele knock–out of ORF 4 in TREU 927 gave a change in phenotype in the transfected cell lines, and this was confirmed by allele knock–in experiments, transferring the resistance allele from the resistant TREU 927 stock into the STIB 247 sensitive stock. The clones derived from these transfected cell lines were then tested for cross–resistance to the arsenicals, MelB, MelOx and MelW, and the diamidine Berenil and the same results were observed as those determined for MelCy i.e. allele knock–out clones were sensitive and allele knock–in clones were resistant (Chapter 6). Therefore, the

locus identified (ORF 4) has been named, *ard* (<u>ar</u>senical and <u>d</u>iamidine) resistance gene.

The *ard* gene encodes a hypothetical protein that has a putative signal peptide/transmembrane domain near the N–terminus, however, further investigation is required to determine the actual function of this domain. Signal peptides and transmembrane domains both contain a stretch of hydrophobic amino acids and this common feature makes it difficult for bio–informatic analysis to distinguish between the two possible functions of such regions. Therefore, the inability to reliably distinguish between N–terminal transmembrane domains and signal peptides limits the prediction of secretory protein status or transmembrane topology. In light of this problem a computational method for discriminating between the two has been established which has been used for bacterial analysis and given very promising results (Yuan *et al.*, 2003). This computational differentiation of N–terminus signal peptides and transmembrane domains is one way that may be used in the future to determine which is present in the ARD protein.

The finding that the *ard* gene plays a role in conferring resistance leads to a range of future experiments. These would lead to the determination of the features of the gene and its products as well as analysing its role in arsenical resistance in the field. There are numerous experiments that could be undertaken to improve our understanding of the mechanism by which *ard* confers resistance. These are discussed below.

The 'knock–out' and 'knock–in' clones, transfecting ORF4 have been screened for phenotype and compared with TREU 927 and STIB 247 using the AlamarBlue screening assay. A comparative study could be undertaken to determine if the same effect on resistance occurs *in vivo*. In order to do this bloodstream form trypanosomes would need to be transfected so that either the resistant allele in TREU 927 was knocked out or the sensitive allele in STIB 247 was replaced with the resistant allele of TREU 927. After electroporation the cells would be allowed to recover for 1hr in Cunninghams medium at 37°C and then used to inoculate mice. The knock–out lines would require to be selected *in vivo* with Geneticin. Then, drug resistance analysis of the transfected cells *in vivo* using a single dose of MelCy for each mouse over a range of concentrations (described in Chapter 4) could then be determined. The analysis would lead to the determination of phenotype of the transfectants and if the results were the same as those found *in vitro*, this would show that the *ard* gene determines arsenical resistance *in vivo*.

An important question is whether the *ard* gene is essential? To answer this, gene knock–out of both alleles of *ard* needs to be carried out. This would be carried out initially on procyclic forms of the resistant TREU 927 stock. In order to knock–out both alleles of *ard*, sequential replacement of the *ard* encoding region with selectable markers would be required. The two selectable drug resistance markers, for example hygromycin and phleomycin, surrounded by *ard* flanking sequence would then be used for transfections into TREU 927 procyclic forms. Following transfections and selection for drug resistance, verification by PCR analysis and a northern blot if *ard* had been

deleted would be required. If *ard* is shown to be an essential gene then it would be expected that the transfected cells would be unable to proliferate and thus would eventually die. It may be necessary to introduce an episomal copy of *ard* before disrupting the second allele. The transformed cells could be cyclically transmitted through tsetse flies that would then be allowed to feed on mice thereby obtaining bloodstream forms. These bloodstream forms could then be tested *in vivo* for their resistance phenotype.

Definitive proof for the association between the resistance phenotype to the *ard* gene could be achieved by episomal transformation. By using an episomal vector transformation this would be independent of the parasite's genome. Therefore, if the resistance phenotype was detected then the activity would be directly attributed to the transformed DNA. Extrachromosomal replication of DNA has been observed in *Trypanosoma cruzi* and *Leishmania* spp. and cosmid vectors have been successfully used in these organisms, but these vectors were unable to replicate in *T. brucei*. A number of different episomes have been described, which replicate autonomously after introduction into *T. brucei* procyclic cells by electroporation. One of these, trypanosome artifical chromosome (TAC) are stably maintained in the absence of drug pressure, but over time the addition of telomeric repeats increase the size of the TACs and so making the recovery and analysis of any cloning products difficult (Sommer *et al.*, 1996). However, it has been shown that DNA isolated from *T. brucei* mitochondrial minicircles allows plasmid DNA to replicate autonomously in the nucleus of *T. brucei* (Metzenberg and Agabian, 1994). Therefore, this DNA fragment can be transferred into a Bluescript-

derived *T. brucei* expression vector, which is the basis for an efficient transfections vector for stable, episomal expression of genes in procyclic cells (Sommer *et al.*, 1996). Confirmation of this would require isolating the episome, therefore the cells would be re-transformed to the parental strain. Thus loss of the episomes should result in complete reversion to the sensitive phenotype of the untransformed line. The transcription levels of episomal *ard* expression and the chromosomal *ard* gene should also be compared using RT-PCR.

In an alternative approach to determine if *ard* was responsible for drug resistance, procyclic forms of STIB 247 could be transfected with the resistant allele of *ard* from TREU 927 expressing the *Tet* repressor therefore, expression of resistance would be mediated by a tetracycline inducible promoter. The construct used to generate the cell lines should also contain a selectable marker for resistance to, for example, hygromycin, thus allowing for selection of transformants. After transfections into trypanosomes, hygromycin-resistant transformants could be selected in the absence of tetracycline. Therefore the inducible promoter would be switched off and it would be expected that the cells would remain sensitive. However, by switching the gene on with the addition of tetracycline to the culture medium it would be expected that the cells would express a resistant phenotype, determined using the AlamarBlue assay.

It would be useful to determine if a transmembrane domain exists as this has implications as to the mode of action and target of the drug within the cell. The action of any anti-parasitic drug is dependent on the efficiency by which it is taken up by the

parasite. We already know that the P2 transporter is involved in arsenical and diamidine uptake but this study has shown that there is another route involved in drug uptake as procyclic forms do not have detectable P2 activity (de Koning, 2001B) yet they are susceptible to the drug. Drug uptake can occur by four main routes, passive diffusion, endocytosis, receptor–mediated uptake or transporter-mediated uptake (de Koning, 2001B) and it is possible that the *ard* gene product is involved in drug uptake. Alternatively, the *ard* gene product could be a specific target or involved in the activation of the drug. It is important therefore, to determine whether the *ard* gene is involved in the uptake of arsenicals and diamidines in order to define its function.

An epitope tag system could also be used to determine which region of the protein contains the sequence necessary for localisation within the cell. This technique has already been used to tag *T. brucei* genes. Epitope tagging of proteins has become a method of choice for the analysis of function, interaction and subcellular localisation of proteins (Shen *et al.*, 2001). A full length copy of the *ard* gene in TREU 927 procyclic forms could be epitope tagged by introducing the Ty1 virus like particle from the well characterised major structural protein of *Saccharomyces cerevisiae* (Bastin *et al.*, 1996). There are two reasons for using this tag, firstly it is recognised by two different monoclonal antibodies (BB2 and TYG5) with overlapping binding sites and secondly, Ty1 is a linear eiptope with a known amino acid sequence and it is recognised in different mutant forms of the protein and therefore it can be recognised in different environments (Bastin *et al.*, 1996).

In order to determine where *ard* localises within the cell, the Ty1 epitope would need to be introduced within the *ard* gene followed by cloning the tagged gene into an inducible expression vector. Transformation of the construct into TREU 927 procyclic forms expressing the tetracycline repressor would then be required so that expression of the tagged *ard* gene was dependent on the presence of tetracycline. After cloning the resulting transformants, by limiting dilution, the tagged cell lines could then be tested for localization within the cell. Cells with the epitope tagged protein, grown in the absence of tetracycline should not be detected by immunofluorescence with the antitag antibody BB2. However, cells that have been grown in the presence of tetracycline for a week then the ARD fusion protein should be detectable by immunofluorescence and should show where the ARD protein has localised within the cell.

A follow on investigation from this would be to truncate the *ard* gene at several points and epitope tag each truncated version for cloning and transfection into wild–type drug sensitive trypanosomes. Therefore, the truncated proteins would be expressed in the presence of the wild-type endogenous ARD. Immunofluorescence of each truncated protein would determine which region of *ard* is necessary for localisation within the cell. An example, where this has been successfully achieved is the finding that the region between amino acids 514–570 of the paraflagellar rod, PFRA, contained sequence necessary but not sufficient for flagellar localisation. This part of the PFRA sequence could therefore act as a target signal or be part of a system involving different regions of the protein (Bastin *et al.*, 1999). Using these approaches would therefore further elucidate on the function of the *ard* gene.

An alternative approach would be to raise an antibody to the expressed protein, followed by immunolocalisation of the ARD protein. It should also be considered that ARD may be associated in a complex with other proteins. As a result, antisera to the *ard* gene product could be produced and immunoprecipitation of the protein extract could be used to detect if the ARD protein forms a complex with other proteins in the cell extract. If ARD is associated with a complex of proteins, then these could be identified by mass spectrometry. As a result this might give important clues as to the function of the gene.

A further approach to the determination of the intracellular target of Cymelarsan might be to assay whether the ARD protein binds to the drug. In order to determine this the *ard* gene would need to be expressed as a recombinant protein followed by affinity chromatography using cymelarsan immobilized on Sepharose beads (Denise *et al.*, 1999). A known amount of the ARD recombinant protein could then be run through the column and the eluate collected. A measure of the amount of protein washing through the column would then be determined and if this was less than the amount originally put on then this would be evidence that the protein (from the *ard* gene product) binds Cymelarsan. Thus, these investigations listed above would lead to the determination of where the ARD protein localises within the cell and if it binds Cymelarsan, which could eventually lead to its determination as a possible drug target.

Determinating which of the four point mutations in *ard* confers the resistance phenotype is required. However, before doing this, these four point mutations need to be confirmed, firstly by PCR followed by cloning of both alleles of TREU 927 and finally sequencing of the cloned products. After confirmation that the four point mutations are

true then using the PCR allele knock-in approach will provide a direct means of evaluating the role of each point mutation. By transfecting each point mutation from TREU 927 into STIB 247, followed by phenotypic analysis of the resulting transfectants will determine which point mutation is involved in conferring the drug resistance phenotype.

One important question that could be addressed is; what is the relevance of these mutations in terms of resistance in the field? In order to achieve this, analysis of the levels of MelCy drug resistance in field isolates is required using the AlamarBlue screening assay on procyclic forms. Cross resistance patterns could then be determined for other arsenicals namely MelB, MelW and MelOx, and also to the diamidines Berenil and Pentamidine. Analysis of bloodstream forms should be undertaken, using either or both the AlamarBlue assay and the growth inhibition assay for all drugs listed above, both *in vitro* and *in vivo* using MelCy. A comparison could then be made between the resistance patterns from field isolates and the isolates derived from this study.

The next step would then be to determine if the point mutations in the *ard* gene, thought to be involved in conferring drug resistance, are present in the field isolates. In order to determine this, amplification of the *ard* gene in the field isolates would be required, followed by cloning of both alleles and sequence analysis of the cloned products. As a result, the sequence comparisons between the field isolates and the isolates from this study would indicate, firstly if there was an association between the mutations found

from this study and those found in the field isolates and secondly, if all four mutations are present in the field isolates or just some of the mutations. The results would also go to prove or disprove the results from the previous investigation as to which of the point mutations is involved in conferring resistance in the isolates derived in this study. As a result, therefore, a direct comparison could be made between the resistance levels found in this study compared to those found from field isolates.

In order to determine if there is any relevance between the level of resistance found in this study compared to field isolates I would have needed to analyse field isolates, which was beyond the scope of this study. The question, however, would be whether the difference in the levels of resistance measures between STIB 247 and STIB386/TREU 927 would lead to treatment failure in the field. There are few reliable measurements of Melarsoprol levels in the bloodstream or brain of treated humans and it is also difficult to correlate the doses administered to humans with those in mice given the possible differences in the pharmacokinetics of the drug between man and a murine model.

In this context however it is interesting to note a study where 19 individuals with late stage *T. b. gambiense* were treated with melarsoprol to determine if a shorter treatment regime was viable. It was found that treatment with ten consecutive doses of 2.6 mg/kg melarsoprol was sufficient to eliminate trypanosomes from the bloodstream (Burri *et al.*, 1993). These doses are similar to those that I have used in mice where 2 – 5 mg/kg MelCy was required to clear infection with STIB 386 and TREU 927.

The cross-resistance observed between the arsenicals and the diamidine Berenil suggested that resistance is mediated by the *ard* gene point mutations and provided direct evidence for the important role for this gene in determining susceptibility to these drugs. A parallel situation has been found in terms of the ability of mutations in the Plasmodium *pfcrt* gene to confer cross resistance to drugs other than chloroquine namely, amodiaquine, quinine, mefloquine and artemisinin, which is also mediated by *pfcrt* point mutations. These finding indicated the need for monitoring resistance, including screening for possible additional changes in *pfcrt* sequence with the increased use of antimalarials (Sidhu *et al.*, 2002). Similar screening should be considered for trypanocides.

The identification of the P2 adenosine transporter (Carter and Fairlamb, 1993) as a key protein in the uptake of the arsenicals and diamidines has led to suggestions that this selective uptake pathway could be used to design new drugs that are selectively imported into the trypanosome. To date no new drugs of this nature are being developed. However, there are ongoing investigations on several novel trypanocides. GB-II-5 and GB-II-150 have been shown to inhibit the assembly of tubulin, so that cells cannot divide, at least *in vitro*. The IC_{50} values for these are 0.4µM for GB-II-5 and 0.05 µM for GB-II-150 using bloodstream forms *in vitro*. No studies have been carried out *in vivo* with GB-II5 and GB-II-150, but these are a new class of tubulin targeted antikinetoplastid drugs (Werbovetz *et al.*, 2003 ASTMH meeting).

Another very promising orally available novel compound, DB289, the prodrug of DB75, is in phase IIA clinical trials. DB289 may be an alternative to Pentamidine as the drug of choice for the treatment of early stage *T. b. gambiense*. This clinical trial is looking very promising as only one relapse out of the 21 patients treated has been observed after a six month post-treatment evaluation. The treatment regime is 100 mg of DB289 orally twice daily for five days. There have been minor side effects reported, headaches and intermittent fevers. Despite this, DB289 is a very promising treatment for early stage *T. b. gambiense* (Burri *et al.*, 2003 ASTMH meeting). One other group of compounds the bisphosphonates, have been proposed to possibly target the trypanosomal acidocalcisome and show considerable promise. These drugs are already in use for the treatment of bone disorders such as osteoporosis, and are well tolerated and achieve parasitological cure in rodents (Barrett *et al.*, 2003). It is clear that there is a major effort by the scientific community to firstly understand the pathways involved in drug resistance that can lead to the development of novel drugs, and secondly suggests that in the near future new drugs may be available for treatment of sleeping sickness.

A good example of the proposed approach for the progression of this study is that of the findings of the relevance of the *pfcrt* genes involvement in resistance in *P. falciparum*. In *P. falciparum* mutations in the *pfcrt* gene, found to confer chloroquine resistance, were present in isolates obtained from Asia, Africa and South America. Sequence analysis revealed consistent association between pfcrt mutations in chloroquine resistant isolates from Asia and Africa for seven of the eight mutations. Sequences of *pfcrt* from South America were also examined and found to contain two of

the mutations found in the Asian and African isolates, namely K76T and A220S (Fidock et al., 2000). The significance of K76T and A220S in chloroquine resistance has been reinforced in a recent clinical trial in West Africa, where these two mutations were found in 100% of persisting infections (Djimde et al., 2001). Obviously the identification of the *pfcrt* gene and its role in chloroquine resistance in field isolates and also the fact that it has been shown to localise in the digestive vacuole makes it a very worthy candidate for drug targeting. Therefore, the sooner field isolates can be screened for the *ard* mutations and localisation investigations carried out the sooner drug targets can be determined.

An example of how the determination of the mechanism of resistance has led to the identification of a novel drug target is provided by the identification of the involvement of the *pfcrt* gene in chloroquine resistance. Antimalarials are believed to act, in part, by complexing with the haem products and it is thought that PfCRT mutations affect accumulation by altering drug flux across the digestive vacuole membrane and may affect drug haematin binding. The discovery of a possible role of haem binding offers alternative strategies for targeting the haemoglobin detoxification pathway for malaria treatment (Sidhu et al., 2002).

The results presented in this thesis have led to the identification of a novel gene that determines resistance to both the arsenicals and the diamidine Berenil as well as establishing the proof of principle that genetic analysis coupled with positional cloning

can be used in *T. brucei* to identify genes of importance for the treatment of human sleeping sickness.

Appendix A1

DNA extraction buffer
10 ml of 1M Tris pH 8

2 ml 0.5M EDTA

4 ml 5M NaCl

Made up to 200 ml with sterile water.

5x TBE electrophoresis buffer
2M Tris

2M Boric acid

20mM EDTA

Made up to 5 litres in distilled water to pH 8 - 8.3

1 M Tris pH8
Tris 121 g dissolved in 800 ml sterile water and pH adjusted to 8 by adding 42 ml

concentrated Hydrochloric acid (HCL) and made up to 1 L with sterile water.

1M Phosphate buffered saline (PBS)
10mM Na_2HPO_4

2mM $KH_2 PO_4$

0.136M NaCl

pH 8

Phosphate buffered saline with glucose (PBSG)
As above with glucose added to make a final concentration of 10mM .

Lysis buffer
50 mM Tris pH 8

100 mM EDTA pH 8

0.5% SDS

Zimmerman Post Transfusion Buffer

132 mM NaCl

8 mM KCL

8 mM Na_2HPO_4

1.5 mM KH_2PO_4

0.5 mM Mg acetate

90 µM CaCl

Table A1.A

SDM – 79 (BRUN MEDIUM)	
Components	**Quantity per litre**
Glucose	1 g
Hepes	8 g
MoPS	5 g
NaHCO$_3$	2 g
Na pyruvate	100 mg
L – Alanine	200 mg
L – Arginine	100 mg
L – Glutamine	300 mg
L – Methionine	70 mg
L – Phenylalanine	80 mg
L – Proline	600 mg
L – Serine	60 mg
L – Taurine	160 mg
L – Threonine	350 mg
L – Tyrosine	100 mg
Adenosine	10 mg
Guanosine	10 mg
Glucosamine – HCL	50 mg
Folic acid	4 mg
p – Amino benzoic acid	2 mg
Biotin	0.2 mg
MEM amino acids non-essential (50x)	8 ml
MEM amino acids (100x)	6 ml
MEM *	7 g
Medium 199**	2 g

Table A1.A: Composition of SDM-79. * MEM should be with Earles salts, without L- glutamine and NaHOH available from Gibco cat no. 072-01700. ** Medium 199 should be with L-glutamine and without NaHCO$_3$ available from Gibco cat no. 071 – 1100.The pH was adjusted to 7.3 and the medium filter sterilized. Before use the medium was supplemented with 10% Foetal calf serum and 2% gentamicin.

Table A1.B

CUNNINGHAMS MEDIUM

Components	Quantity per 100 ml
Inorganic Salts	
NaH_2PO_4	53 mg
$MgCl_26H_2O$	304 mg
$MgSo_4\ 7H_2O$	370 mg
KCL (anhydrous)	298 mg
$CaCl_22H_2O$	15 mg
Sugars	
Glucose	70 mg
Fructose	40 mg
Sucrose	40 mg
Organic Acids	
L – Malic	67 mg
α – Ketoglutaric	37 mg
Fumaric	5.5 mg
Succinic	6 mg
Amino Acids	
β – Alanine	200 mg
DL – Alanine	109 mg
L – Arginine	44 mg
L – Asparagine	24 mg
L – Aspartic acid	11 mg
L – Cysteine HCL	8 mg
L – Cysteine	3 mg
L – Glutamic acid	25 mg
L – Glutamine	164 mg
Glycine	12 mg

Table A1.B Cont

CUNNINGHAMS MEDIUM (cont)

Components	Quantity per 100ml
L – Histidine	16mg
DL – Isoleucine	9mg
L – Leucine	9mg
L – Lysine	15mg
DL – Methionine	20mg
L – Phenylalanine	20mg
L – Proline	690mg
DL – Serine	20mg
L – Taurine	27mg
DL – Threonine	10mg
L – Tryptophan	10mg
L – Tyrosine	20mg
DL – Valine	21mg
Vitamin Mixture BME (100x)	0.2ml
Indicator Phenol Red (0.5%)	0.4ml

Table A1.B: Components of Cunningham Medium. The compounds were dissolved in double distilled water as follows: Inorganic salts in 10 ml except $CaCl_2$, which was in 5 ml. Sugars in 10 ml, Organic acids in 5 ml and Amino acids in 65 ml. The Vitamin mixture BME (100x) and Indicator phenol red was added and made up to 100 ml at pH 7.4. The mixture was filter sterilized and stored at -20°C. Foetal calf serum and 2% gentamycin was added at a concentration of 30% before use.

Table A2.A *In vitro* cloning of STIB 247 x TREU 927

Identification	Markers					Comments
	CRAM	292	MS42	JS2	PLC	
Parental 247	1 - 1	5 - 5	5 - 5	5 - 6	5 - 5	
Parental 927	3 - 4	3 - 4	3 - 4	3 - 4	3 - 4	
Procyclic Cloning						
Cross 247 x 927						
Batch 1						
F532/72 pcl 1 - 118	1 - 3 - 4	3 - 4 - 5	3 - 4 - 5	3 - 4 - 5 - 6	3 - 4 - 5	Mixed/Trisomic/ Triploid
Batch 2						
F532/72 pcl 1 - 52	1 - 3	4 - 5	4 - 5	3 - 5	4 - 5	1 New Genotype
Batch 3						
F532/72 pcl 1 - 76	1 - 3	3 - 5	3 - 5	4 - 5	4 - 5	All same genotype as F532/72 mcl 6
Batch 4						
F532/72 pcl 1	1 - 4	4 - 5	3 - 5	3 - 5	3 - 5	Same as F532/72 mcl 1
F532/72 pcl 2	1 - 4	3 - 5	3 - 5	3 - 5	3 - 5	Replaced with F532/72 bscl 1
F532/72 pcl 3	1 - 4	4 - 5	4 - 5	4 - 6	3 - 5	Same as F532/72 mcl 7
F532/72 pcl 4	1 - 4	3 - 5	3 - 5	3 - 5	3 - 5	Replaced with F532/72 bscl 1
F532/72 pcl 5	1 - 3	4 - 5	3 - 5	4 - 5	3 - 5	New Genotype
F532/72 pcl 6	1 - 3	4 - 5	3 - 4 - 5	3 - 6	3 - 4 - 5	Mixed/ Trisomic
F532/72 pcl 7	1 - 4	3 - 5	3 - 5	4 - 6	3 - 5	New Genotype
F532/72 pcl 8	1 - 3	4 - 5	3 - 5	4 - 6	F	New Genotype
F532/72 pcl 9	1 - 4	3 - 4 - 5	4 - 5	3 - 5 - 6	3 - 5	Mixed/Trisomic
F532/72 pcl 10	1 - 3	3 - 5	3 - 5	4 - 5	4 - 5	Same as F532/72 mcl 6
F532/72 pcl 11	1 -3	3 - 5	3 - 4 - 5	4 - 6	3 - 5	Mixed/Trisomic
F532/72 pcl 12	1 - 4	3 - 5	3 - 5	4 - 5	4 - 5	Replaced with F532/72 bscl 2
F532/72 pcl 13	1 - 4	3 - 5	3 - 5	3 - 5	3 - 5	Replaced with F532/72 bscl 1
F532/72 pcl 14	1 - 4	3 - 5	3 - 5	4 - 5	4 - 5	Replaced with F532/72 bscl 2
F532/72 pcl 15	1 - 4	3 - 5	3 - 5	3 - 5	3 - 5	Replaced with F532/72 bscl 1
F532/72 pcl 16	1 - 4	4 - 5	3 - 5	4 - 5	4 - 5	Same as F532/72 mcl 2
F532/72 pcl 17	1 - 4	3 - 5	3 - 5	4 - 5	4 - 5	Replaced with F532/72 bscl 2
F532/72 pcl 18	1 - 4	3 - 5	3 - 5	3 - 5	3 - 5	Replaced with F532/72 bscl 1
F532/72 pcl 19	1 - 4	3 - 5	4 - 5	4 - 5	3 - 5	Same as F532/72 mcl 8
F532/72 pcl 20	1 - 4	3 - 5	3 - 5	3 - 5	3 - 5	Replaced with F532/72 bscl 1
F532/72 pcl 21	1 - 4	4 - 5	4 - 5	4 - 5	3 - 5	Same as F532/72 mcl 3

Table A2.A Cont *In vitro* cloning of STIB 247 x TREU 927

Identification	Markers					Comments
	CRAM	292	MS42	JS2	PLC	
Parental 247	1 - 1	5 - 5	5 - 5	5 - 6	5 - 5	
Parental 927	3 - 4	3 - 4	3 - 4	3 - 4	3 - 4	
Procyclic Cloning Cross 247 x 927						
Batch 4 Cont						
F532/72 pcl 22	1 - 4	3 - 5	3 - 5	3 - 5	3 - 5	Replaced with F532/72 bscl 1
F532/72 pcl 23	1 - 4	3 - 5	3 - 5	4 - 5	4 - 5	Replaced with F532/72 bscl 2
F532/72 pcl 24	1 - 3	3 - 5	3 - 5	4 - 5	4 - 5	Same as F532/72 mcl 6
F532/72 pcl 25	1 - 4	3 - 5	3 - 5	3 - 5	3 - 5	Replaced with F532/72 bscl 1
F532/72 pcl 26	1 - 3	3 - 5	3 - 4 - 5	3 - 6	3 - 4 - 5	Mixed/Trisomic
F532/72 pcl 27	1 - 4	4 - 5	4 - 5	3 - 5	3 - 5	Same as F532/72 mcl 5
F532/72 pcl 28	1 - 4	3 - 5	3 - 5	3 - 5	3 - 5	Replaced with F532/72 bscl 1
F532/72 pcl 29	1 - 3	4 - 5	4 - 5	3 - 5	4 - 5	Same as batch 2, F532/72 pcl1
F532/72 pcl 30	1 - 4	4 - 5	3 - 5	3 - 5	3 - 5	Same as F532/72 mcl 1
F532/72 pcl 31	1 - 4	3 - 5	3 - 5	3 - 5	3 - 5	Replaced with F532/72 bscl 1
F532/72 pcl 32	1 - 4	3 - 5	3 - 5	3 - 5	3 - 5	Replaced with F532/72 bscl 1
F532/72 pcl 33	1 - 4	3 - 5	3 - 5	4 - 5	4 - 5	Replaced with F532/72 bscl 2
F532/72 pcl 34	1 - 3	3 - 4 - 5	4 - 5	3 - 5	3 - 4 - 5	Mixed/Trisomic
F532/72 pcl 35	1 - 4	3 - 5	3 - 5	4 - 5	4 - 5	Replaced with F532/72 bscl 2
F532/72 pcl 36	1 - 3	3 - 4 - 5	4 - 5	4 - 6	3 - 4 - 5	Mixed /Trisomic
F532/72 pcl 37	1 - 4	3 - 5	3 - 5	3 - 5	3 - 5	Replaced with F532/72 bscl 1
F532/72 pcl 38	1 - 4	3 - 5	3 - 5	4 - 5	4 - 5	Replaced with F532/72 bscl 2
F532/72 pcl 39	1 - 4	4 - 5	4 - 5	3 - 5	3 - 5	Same as F532/72 mcl 6
F532/72 pcl 40	1 - 4	3 - 5	3 - 5	4 - 5	4 - 5	Replaced with F532/72 bscl 2

Table A2.A Cont *In vitro* cloning of STIB 247 x TREU 927

Identification	Markers					Comments
	CRAM	292	MS42	JS2	PLC	
Parental 247	1 - 1	5 - 5	5 - 5	5 - 6	5 - 5	
Parental 927	3 - 4	3 - 4	3 - 4	3 - 4	3 - 4	
Procyclic Cloning						
Cross 247 x 927						
Batch 4 Cont						
F532/72 pcl 41	1 - 3	3 - 4 - 5	4 - 5	3 - 5	3 - 5	Mixed/Trisomic
F532/72 pcl 42	1 - 4	3 - 5	3 - 5	3 - 5	3 - 5	Replaced with F532/72 bscl 1
F532/72 pcl 43	1 - 4	3 - 5	3 - 5	4 - 5	4 - 5	Replaced with F532/72 bscl 2
F532/72 pcl 44	1 - 4	3 - 5	3 - 5	3 - 5	3 - 5	Replaced with F532/72 bscl 1
F532/72 pcl 45	1 - 4	3 - 5	3 - 5	4 - 5	4 - 5	Replaced with F532/72 bscl 2
F532/72 pcl 46	1 - 3	4 - 5	3 - 5	3 - 4 - 5	3 - 4 - 5	Mixed/Trisomic
F532/72 pcl 47	1 - 4	4 - 5	4 - 5	3 - 6	3 - 5	Same as F532/72 mcl 9
F532/72 pcl 48	1 - 4	4 - 5	4 - 5	4 - 5	3 - 5	Same as F532/72 mcl 3
F532/72 pcl 49	1 - 4	3 - 5	3 - 5	4 - 5	4 - 5	Replaced with F532/72 bscl 2
F532/72 pcl 50	1 - 4	4 - 5	3 - 5	4 - 5	4 - 5	Same as F532/72 mcl 2
F532/72 pcl 51	1 - 4	3 - 5	3 - 5	4 - 6	3 - 5	Same as F532/72 pcl 7
F532/72 pcl 52	1 - 4	3 - 5	3 - 5	3 - 5	3 - 5	Replaced with F532/72 bscl 1
F532/72 pcl 53	1 - 3	4 - 5	3 - 5	4 - 5	3 - 5	Same as F532/72 pcl 5
F532/72 pcl 54	1 - 4	4 - 5	4 - 5	3 - 6	3 - 5	Same as F532/72 mcl 9
F532/72 pcl 55	1 - 3	3 - 4 - 5	4 - 5	4 - 5	4 - 5	Mixed/Trisomic
F532/72 pcl 56	1 - 3	4 - 5	4 - 5	3 - 5	4 - 5	Same as batch 2, F532/72 pcl 1
F532/72 pcl 57	1 - 4	4 - 5	3 - 4 - 5	4 - 5	3 - 4 - 5	Mixed/Trisomic
F532/72 pcl 58	1 - 4	3 - 5	3 - 5	4 - 6	3 - 5	Same as F532/72 pcl 7
F532/72 pcl 59	1 - 4	4 - 5	3 - 5	3 - 5	3 - 5	Same as F532/72 mcl 1
F532/72 pcl 60	1 - 3	3 - 4 - 5	3 - 5	4 - 6	4 - 5	Mixed/Trisomic
F532/72 pcl 61	1 - 4	4 - 5	4 - 5	4 - 5	3 - 5	Same as F532/72 mcl 3
F532/72 pcl 62	1 - 4	3 - 5	3 - 5	3 - 5	3 - 5	Replaced with F532/72 bscl 1
F532/72 pcl 63	1 - 3	4 - 5	3 - 5	4 - 5	3 - 5	Same as F532/72 pcl 5
F532/72 pcl 64	1 - 4	3 - 5	3 - 4 - 5	3 - 6	3 - 4 - 5	Mixed/Trisomic
F532/72 pcl 65	1 - 4	3 - 5	3 - 5	4 - 5	4 - 5	Replaced with F532/72 bscl 2

Table A2.A Cont *In vitro* cloning of STIB 247 x TREU 927

Identification	Markers					Comments
	CRAM	**292**	**MS42**	**JS2**	**PLC**	
Parental 247	1 - 1	5 - 5	5 - 5	5 - 6	5 - 5	
Parental 927	3 - 4	3 - 4	3 - 4	3 - 4	3 - 4	
Procyclic Cloning Cross 247 x 927						
Batch 4 Cont						
F532/72 pcl 66	1 - 4	4 - 5	3 - 5	4 - 5	4 - 5	Same as F532/72 mcl 2
F532/72 pcl 67	1 - 3	4 - 5	3 - 4 - 5	4 - 6	3 - 4 - 5	Mixed/Trisomic
F532/72 pcl 68	1 - 4	3 - 5	3 - 5	4 - 5	4 - 5	Replaced with F532/72 bscl 2
F532/72 pcl 69	1 - 4	3 - 5	3 - 5	4 - 6	3 - 5	Same as F532/72 pcl 7
F532/72 pcl 70	1 - 4	3 - 4 - 5	4 - 5	3 - 5	4 - 5	Mixed/Trisomic
F532/72 pcl 71	1 - 3	4 - 5	4 - 5	3 - 5	4 - 5	Same as batch 2, F532/72 pcl 1
F532/72 pcl 72	1 - 4	3 - 5	3 - 5	3 - 5	3 - 5	Replaced with F532/72 bscl 1
F532/72 pcl 73	1 - 3	4 - 5	3 - 5	4 - 5	3 - 5	Same as F532/72 pcl 5
F532/72 pcl 74	1 - 4	3 - 5	4 - 5	4 - 6	3 - 4 - 5	Mixed/Trisomic
F532/72 pcl 75	1 - 4	4 - 5	4 - 5	3 - 6	3 - 5	Same as F532/72 mcl 9
F532/72 pcl 76	1 - 3	3 - 4 - 5	3 - 4 - 5	3 - 5	4 - 5	Mixed/Trisomic
F532/72 pcl 77	1 - 4	3 - 4 - 5	3 - 5	3 - 6	3 - 5	Mixed/Trisomic
F532/72 pcl 78	1 - 3	4 - 5	4 - 5	3 - 5	4 - 5	Same as batch 2, F532/72 pcl 1
F532/72 pcl 79	1 - 4	3 - 5	3 - 5	3 - 5	3 - 5	Replaced with F532/72 bscl 1
F532/72 pcl 80	1 - 4	4 - 5	4 - 5	4 - 5	3 - 5	Same as F532/72 mcl 3
F532/72 pcl 81	1 - 4	4 - 5	3 - 5	3 - 4 - 5	3 - 4 - 5	Mixed/Trisomic
F532/72 pcl 82	1 - 4	3 - 5	4 - 5	4 - 5	3 - 5	Same as F532/72 mcl 8
F532/72 pcl 83	1 - 4	3 - 5	3 - 5	4 - 5	3 - 4 - 5	Mixed/Trisomic
Batch 5						
F532/72 pcl 1 - 121	1 - 4	3 - 5	3 - 5	3 - 5	3 - 5	All same genotype, Replaced with F532/72 bscl 1

Table A2.B *In vivo* cloning STIB 247 x TREU 927

Identification	Markers					Comments
	CRAM	292	MS42	JS2	PLC	
Parental 247	1 - 1	5 - 5	5 - 5	5 - 6	5 - 5	
Parental 927	3 - 4	3 - 4	3 - 4	3 - 4	3 - 4	
Bloodstream Cloning						
Cross 247 x 927						
F532/72						
F532/72 bscl 1	1 - 4	3 - 5	3 - 5	3 - 5	3 - 5	New Genotype
F532/72 bscl 2	1 - 4	3 - 5	3 - 5	4 - 5	4 - 5	New Genotype
F532/72 bscl 3	1 - 4	3 - 4 - 5	3 - 5	3 - 5	3 - 5	Mixed/Trisomic
F532/72 bscl 4	1 - 4	4 - 5	3 - 5	3 - 5	3 - 5	Same as F532/72 mcl 1
F532/72 bscl 5	1 - 3	3 - 5	3 - 5	4 - 5	4 - 5	Same as F532/72 mcl 6
F532/72 bscl 6	1 - 4	3 - 5	3 - 5	3 - 4 - 5	4 - 5	Mixed/Trisomic
F532/72 bscl 7	1 - 4	3 - 4 - 5	3 - 5	4 - 5	3 - 5	Mixed/Trisomic
F532/72 bscl 8	1 - 3	3 - 5	3 - 5	4 - 5	4 - 5	Same as F532/72 mcl 6
F532/72 bscl 9	1 - 4	3 - 4 - 5	3 - 5	3 - 5	4 - 5	Mixed/Trisomic
F532/72 bscl 10	1 - 4	4 - 5	3 - 4 - 5	3 - 5	4 - 5	Mixed/Trisomic
F532/72 bscl 11	1 - 4	3 - 5	3 - 5	3 - 5	3 - 5	Same as F532/72 bscl 1
F532/72 bscl 12	1 - 4	3 - 5	3 - 5	3 - 5	3 - 5	Same as F532/72 bscl 1
F532/72 bscl 13	1 - 4	3 - 4 - 5	3 - 5	3 - 5	4 - 5	Mixed/Trisomic
F532/72 bscl 14	1 - 3	3 - 4 - 5	3 - 5	3 - 5	3 - 5	Mixed/Trisomic
F532/72 bscl 15	1 - 4	3 - 5	3 - 5	4 - 5 - 6	3 - 5	Mixed/Trisomic
F532/72 bscl 16	1 - 4	3 - 5	3 - 5	4 - 5	4 - 5	Same as F532/72 bscl 2
F532/72 bscl 17	1 - 4	3 - 5	3 - 5	3 - 5	3 - 5	Same as F532/72 bscl 1
F532/72 bscl 18	1 - 4	3 - 5	3 - 5	3 - 5	3 - 5	Same as F532/72 bscl 1
Batch 1 F124/28						
F124/28 bscl 1	1 - 3	4 - 5	3 - 5	4 - 6	3 - 5	New Genotype
F124/28 bscl 2	1 - 4	4 - 5	4 - 5	4 - 5 - 6	4 - 5	Mixed/Trisomic
F124/28 bscl 3	1 - 3	3 - 5	4 - 5	4 - 5	4 - 5	New Genotype
F124/28 bscl 4	3 - 4	3 - 4	3 - 4	3 - 4	3 - 4	Parental
F124/28 bscl 5	1 - 4	3 - 5	4 - 5	3 - 5	4 - 5	New Genotype
F124/28 bscl 6	1 - 3	4 - 5	3 - 5	4 - 6	3 - 5	Same as F124/28 bscl 1
Batch 2 F124/28						
F124/28 bscl 1	1 - 3 - 4	4 - 5	3 - 4 - 5	3 - 4 - 5	4 - 5	Mixed/Trisomic
F124/28 bscl 2	1 - 3 - 4	3 - 4 - 5	3 - 4 - 5	3 - 4 - 5	4 - 5	Mixed/Trisomic
F124/28 bscl 3	1 - 4	4 - 5	3 - 4 - 5	3 - 4 - 5	4 - 5	Mixed/Trisomic
F124/28 bscl 4	1 - 3	3 - 4 - 5	3 - 4 - 5	3 - 4 - 5	3 - 4 - 5	Mixed/Trisomic
F124/28 bscl 5	1 - 4	3 - 4 - 5	3 - 4 - 5	3 - 4 - 5	3 - 4 - 5	Mixed/Trisomic
F124/28 bscl 6	1 - 4	3 - 4 - 5	3 - 4 - 5	3 - 4 - 5	4 - 5	Mixed/Trisomic

Table A2.B Cont *In vivo* cloning STIB 247 x TREU 927

| Identification | Markers | | | | | Comments |
	CRAM	292	MS42	JS2	PLC	
Parental 247	1 - 1	5 - 5	5 - 5	5 - 6	5 - 5	
Parental 386	1 - 2	1 - 2	1 - 2	1 - 2	1 - 2	
Parental 927	3 - 4	3 - 4	3 - 4	3 - 4	3 - 4	
Bloodstream Cloning						
Cross 247 x 927						
Batch 2 F124/28						
F124/28 bscl 7	1 - 3	3 - 4 - 5	3 - 4 - 5	3 - 4 - 5	4 - 5	Mixed/Trisomic
F124/28 bscl 8	1 - 4	3 - 4 - 5	3 - 4 - 5	3 - 4 - 5	3 - 4 - 5	Mixed/Trisomic
F124/28 bscl 9	1 - 4	3 - 5	3 - 5	3 - 6	3 - 5	New Genotype
F124/28 bscl 10	1 - 3	3 - 4 - 5	3 - 4 - 5	3 - 4 - 5	3 - 5	Mixed/Trisomic
F124/28 bscl 11	1 - 3	4 - 5	3 - 5	4 - 6	3 - 5	Same as Batch 1 F124/28 bscl 1
F124/28 bscl 12	1 - 4	3 - 5	3 - 5	4 - 6	3 - 5	New Genotype
F124/28 bscl 13	1 - 3	3 - 5	3 - 5	4 - 6	3 - 5	New Genotype
F124/28 bscl 14	1 - 4	3 - 5	4 - 5	3 - 5	3 - 5	New Genotype
F124/28 bscl 15	1 - 3	4 - 5	4 - 5	4 - 5	4 - 5	New Genotype
F124/28 bscl 16	1 - 3	3 - 4 - 5	3 - 4 - 5	4 - 6	3 - 4 - 5	Mixed/Trisomic
F124/28 bscl 17	1 - 4	3 - 4 - 5	3 - 4 - 5	3 - 4 - 5	3 - 5	Mixed/Trisomic
F124/28 bscl 18	1 - 4	3 - 5	4 - 5	4 - 6	4 - 5	Same as F124/28 bscl C5
F124/28 bscl 19	1 - 4	3 - 5	4 - 5	4 - 6	4 - 5	Same as F124/28 bscl C5
F124/28 bscl 20	1 - 3	3 - 5	3 - 5	4 - 5	F	New Genotype
F124/28 bscl 21	1 - 3	3 - 4 - 5	3 - 4 - 5	4 - 5	4 - 5	Mixed/Trisomic
F124/28 bscl 22	1 - 4	4 - 5	4 - 5	4 - 5	4 - 5	New Genotype
F124/28 bscl 23	1 - 4	3 - 4 - 5	3 - 4 - 5	3 - 4 - 5	3 - 4 - 5	Mixed/Trisomic
F124/28 bscl 24	1 - 4	3 - 4 - 5	3 - 4 - 5	3 - 4 - 5	3 - 4 - 5	Mixed/Trisomic
F124/28 bscl 25	1 - 3	3 - 4 - 5	3 - 4 - 5	3 - 4 - 5	3 - 4 - 5	Mixed/Trisomic
F124/28 bscl 26	1 - 4	3 - 4 - 5	3 - 4 - 5	3 - 4 - 5	3 - 4 - 5	Mixed/Trisomic
F124/28 bscl 27	1 - 4	3 - 5	4 - 5	3 - 5	4 - 5	Same as Batch 1 F124/28 bscl 5
F124/28 bscl 28	1 - 4	4 - 5	4 - 5	4 - 5	4 - 5	Same as F124/28 bscl 22
F974/78						
F974/78 bscl 1	3 - 4	3 - 4	3 - 4	3 - 4	3 - 4	Parental
F974/78 bscl 2	3 - 4	3 - 4	3 - 4	3 - 4	3 - 4	Parental
F974/78 bscl 3	1 - 4	4 - 5	3 - 5	4 - 5	4 - 5	New Genotype
F974/78 bscl 4	3 - 4	3 - 4	3 - 4	3 - 4	3 - 4	Parental
F974/78 bscl 5	3 - 4	3 - 4	3 - 4	3 - 4	3 - 4	Parental
F974/78 bscl 6	1 - 3	4 - 5	4 - 5	4 - 6	3 - 5	New Genotype
F974/78 bscl 7	1 - 4	3 - 5	3 - 5	4 - 5	4 - 5	New Genotype
F974/78 bscl 8	1 - 4	4 - 5	3 - 5	4 - 5	4 - 5	Same as F974/78 bscl 3

Table A2.B Cont *In vivo* cloning STIB 247 x TREU 927

Identification	Markers					Comments
	CRAM	**292**	**MS42**	**JS2**	**PLC**	
Parental 247	1 - 1	5 - 5	5 - 5	5 - 6	5 - 5	
Parental 927	3 - 4	3 - 4	3 - 4	3 - 4	3 - 4	
Bloodstream Cloning						
in vivo						
Cross 247 x 927						
F974/78 Cont						
F974/78 bscl 9	1 - 3	4 - 5	4 - 5	4 - 6	3 - 5	Same as F974/78 bscl 6
Re-cloning of F532/72 bscl 15						
F532/72 bscl 15/1B	1 - 4	3 - 5	3 - 5	3 - 5	3 - 5	Same as F532/72 bscl 1
F532/72 bscl 15/2B	1 - 3	3 - 5	3 - 5	4 - 5	4 - 5	Same as F532/72 mcl 6
F532/72 bscl 15/3B	1 - 4	3 - 5	3 - 5	4 - 5	4 - 5	Same as F532/72 bscl 2
F532/72 bscl 15/4B	1 - 4	3 - 5	3 - 5	3 - 5	3 - 5	Same as F532/72 bscl 1
F532/72 bscl 15/5B	1 - 3	3 - 5	3 - 5	4 - 6	3 - 5	New Genotype
F532/72 bscl 15/6B	1 - 4	3 - 5	3 - 5	3 - 5	3 - 5	Same as F532/72 bscl 1
F532/72 bscl 15/7B	1 - 3	3 - 5	3 - 5	4 - 6	3 - 5	Same as F532/72 bscl 15/5B

Table A2.C *In vivo* cloning STIB 247 x STIB 386

Identification	Markers					Comments
	CRAM	292	MS42	JS2	PLC	
Parental 247	1 - 1	5 - 5	5 - 5	5 - 6	5 - 5	
Parental 386	1 - 2	1 - 2	1 - 2	1 - 2	1 - 2	
Bloodstream						
Cloning						
Cross 247 x 386						
F9/41						
F9/41 bscl 1	1 - 1	2 - 5	2 - 5	1 - 6	1 - 5	New Genotype
F9/41 bscl 2	1 - 1	1 - 5	1 - 5	1 - 6	1 - 5	New Genotype
F9/41 bscl 3	1 - 1	1 - 5	1 - 5	1 - 6	1 - 5	Same as F9/41 bscl 2
F9/41 bscl 4	1 - 1	2 - 5	2 - 5	1 - 6	1 - 5	Same as F9/41 bscl 1
F9/41 bscl 5	1 - 2	2 - 5	2 - 5	1 - 5	2 - 5	New Genotype
F9/41 bscl 6	1 - 2	2 - 5	2 - 5	1 - 2 - 5 - 6	1 - 2 - 5	Mixed/Trisomic
F9/41 bscl 7	1 - 2	2 - 5	2 - 5	1 - 5	1 - 5	New Genotype
F9/41 bscl 8	1 - 1	2 - 5	1 - 5	1 - 5	1 - 5	New Genotype
F9/41 bscl 9	1 - 1	1 - 5	1 - 5	2 - 6	2 - 5	New Genotype
F9/41 bscl 10	1 - 2	2 - 5	2 - 5	1 - 5	1 - 5	Same as F9/41 bscl 7
F9/41 bscl 11	1 - 2	1 - 5	1 - 5	1 - 5	2 - 5	New Genotype
F19/31						
F19/31 bscl 1	1 - 2	2 - 5	1 - 5	1 - 6	1 - 5	New Genotype
F19/31 bscl 2	1 - 2	1 - 2 - 5	1 - 5	1 - 6	2 - 5	Mixed/Trisomic
F19/31 bscl 3	1 - 2	1 - 5	1 - 5	2 - 6	2 - 5	Same as F19/31 bscl 10
F19/31 bscl 4	1 - 2	2 - 5	1 - 5	1 - 6	1 - 5	Same as F19/31 bscl 1
F19/31 bscl 5	1 - 1	1 - 5	1 - 5	1 - 6	2 - 5	New Genotype
F19/31 bscl 6	1 - 1	1 - 5	1 - 5	1 - 6	2 - 5	Same as F19/31 bscl 5
F19/31 bscl 7	1 - 2	2 - 5	1 - 5	1 - 6	1 - 5	Same as F19/31 bscl 1
F19/31 bscl 8	1 - 2	1 - 5	1 - 5	2 - 5	2 - 5	New Genotype
F19/31 bscl 9	1 - 1	1 - 5	1 - 5	1 - 6	2 - 5	Same as F19/31 bscl 5
F19/31 bscl 10	1 - 2	1 - 5	1 - 5	2 - 6	2 - 5	New Genotype
F19/31 bscl 11	1 - 1	1 - 5	1 - 5	1 - 5	2 - 5	New Genotype
F19/31 bscl 12	1 - 1	1 - 5	1 - 5	1 - 6	2 - 5	Same as F19/31 bscl 5
F19/31 bscl 13	1 - 2	1 - 5	1 - 5	2 - 6	2 - 5	Same as F19/31 bscl 10
F19/31 bscl 14	1 - 2	1 - 5	1 - 5	2 - 5	2 - 5	Same as F19/31 bscl 8

Table A2.C Cont *In vivo* cloning STIB 247 x STIB 386

	Markers					
Identification	**CRAM**	**292**	**MS42**	**JS2**	**PLC**	**Comments**
Parental 247	1 - 1	5 - 5	5 - 5	5 - 6	5 - 5	
Parental 386	1 - 2	1 - 2	1 - 2	1 - 2	1 - 2	
Bloodstream Cloning						
Cross 247 x 386						
F28/46						
F28/46 bscl 1	1 - 1	1 - 5	1 - 5	2 - 5	1 - 5	New Genotype
F28/46 bscl 2	1 - 1	1 - 5	1 - 5	2 - 5	1 - 5	Same as F28/46 bscl 1
F28/46 bscl 3	1 - 1	1 - 5	1 - 5	2 - 5	1 - 5	Same as F28/46 bscl 1
F28/46 bscl 4	1 - 2	1 - 5	1 - 5	2 - 6	2 - 5	New Genotype
F28/46 bscl 5	1 - 1	1 - 5	1 - 5	2 - 5	1 - 5	Same as F28/46 bscl 1
F28/46 bscl 6	1 - 2	1 - 5	1 - 5	2 - 5	2 - 5	New Genotype
F28/46 bscl 7	1 - 1	2 - 5	1 - 5	2 - 5	1 - 5	New Genotype
F28/46 bscl 8	1 - 2	2 - 5	2 - 5	2 - 6	2 - 5	New Genotype
F28/46 bscl 9	1 - 2	2 - 5	2 - 5	2 - 6	2 - 5	Same as F28/46 bscl 8
F28/46 bscl 10	1 - 2	1 - 5	1 - 5	2 - 5	2 - 5	Same as F28/46 bscl 6
F28/46 bscl 11	1 - 2	2 - 5	2 - 5	2 - 5	2 - 5	New Genotype
F29/46						
F29/46 bscl 1	1 - 1	2 - 5	1 - 5	1 - 5	1 - 5	New Genotype
F29/46 bscl 2	1 - 1	1 - 5	1 - 5	2 - 6	2 - 5	New Genotype
F29/46 bscl 3	1 - 1	1 - 5	1 - 5	2 - 6	2 - 5	New Genotype
F29/46 bscl 4	1 - 1	1 - 5	1 - 5	2 - 6	1 - 5	New Genotype
F29/46 bscl 5	1 - 1	1 - 5	1 - 5	2 - 6	2 - 5	Same as F29/46 bscl 3
F57/40						
F57/40 bscl 1	1 - 2	1 - 2	1 - 2	1 - 2	1 - 2	Parental
F57/40 bscl 2	1 - 2	1 - 2	1 - 2	1 - 2	1 - 2	Parental
F57/40 bscl 3	1 - 2	1 - 2	1 - 2	1 - 2	1 - 2	Parental

Table A2.A–C: Minisatellite analysis of all trypanosome clones derived from genetic crosses. (A) Cross STIB 247 x TREU 927 procyclic clones (pcl). **(B)** Cross STIB 247 x TREU 927 bloodstream clones (bscl). **(C)** Cross STIB 247 x STIB 386 bloodstream clones (bscl). Column 1 indicates the hybrid clone identification number used throughout this study. Columns 2–6 give the results of the mini– and microsatellite analysis. Alleles are numbered 1–5 for the minisatellites CRAM, 292, MS42 and PLC, and 1-6 for the microsatellite JS2. Column 7 gives the interpretation of the result for each clone. All clones that are genotypically unique are shaded. Clones exhibiting three band patterns for one or more markers were considered either a mix or trisomic.

Table A3. A: *In vivo* screening of the three parental lines for the Cymelarsan resistance phenotype

Dose MelCy	PARASITAEMIA													
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
0.1 mg/kg														
STIB 247	o	0	5.7	7.2	7.5	<xsn								
STIB 247	o	0	0	6.9	7.2	8.1	<xsn							
STIB 247	o	5.7	6.9	7.5	<xsn									
STIB 247	o	0	5.7	6.9	7.2	<xsn								
STIB 247	o	5.7	6.9	7.5	<xsn									
0.2 mg/kg														
STIB 247	o	5.7	6.9	7.2	7.5	<xsn								
STIB 247	o	5.7	6.9	7.2	8.1	<xsn								
STIB 247	o	5.7	7.2	8.1	<xsn									
STIB 247	o	o	o	o	o	o	o	o	o	o	o	o	o	o
STIB 247	o	o	o	o	o	o	o	o	o	o	o	o	o	o
0.5 mg/kg														
STIB 247	o	o	o	o	o	o	o	o	o	o	o	o	o	o
STIB 247	o	o	o	o	o	o	o	o	o	o	o	o	o	o
STIB 247	o	o	o	o	o	o	o	o	o	o	o	o	o	o
STIB 247	o	o	o	o	o	o	o	o	o	o	o	o	o	o
STIB 247	o	o	o	o	o	o	o	o	o	o	o	o	o	o
1 mg/kg														
STIB 247	o	o	o	o	o	o	o	o	o	o	o	o	o	o
STIB 247	o	o	o	o	o	o	o	o	o	o	o	o	o	o
STIB 247	o	o	o	o	o	o	o	o	o	o	o	o	o	o
STIB 247	o	o	o	o	o	o	o	o	o	o	o	o	o	o
STIB 247	o	o	o	o	o	o	o	o	o	o	o	o	o	o
2 mg/kg														
STIB 247	o	o	o	o	o	o	o	o	o	o	o	o	o	o
STIB 247	o	o	o	o	o	o	o	o	o	o	o	o	o	o
STIB 247	o	o	o	o	o	o	o	o	o	o	o	o	o	o
STIB 247	o	o	o	o	o	o	o	o	o	o	o	o	o	o
STIB 247	o	o	o	o	o	o	o	o	o	o	o	o	o	o
CONTROL														
STIB 247	o	0	6.9	7.5	<xsn									
STIB 247	o	5.7	6.9	7.5	<xsn									

Table A3. A Cont: *In vivo* screening of the three parental lines for Cymelarsan resistance

Dose MelCy		PARASITAEMIA												
0.2 mg/kg	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
STIB 386	0	0	5.7	7.2	8.1	<xsn								
STIB 386	0	0	5.7	7.2	7.5	<xsn								
STIB 386	0	6.9	7.5	<xsn										
STIB 386	0	0	6.9	7.2	8.1	<xsn								
STIB 386	0	5.7	6.9	7.5	<xsn									
0.5 mg/kg														
STIB 386	0	0	0	0	0	0	0	0	0	0	0	0	0	0
STIB 386	0	5.7	6.9	7.2	7.5	<xsn								
STIB 386	0	0	6.9	7.5	<xsn									
STIB 386	0	5.7	6.9	7.2	7.5	<xsn								
STIB 386	0	0	5.7	6.9	7.5	xsn								
1 mg/kg														
STIB 386	0	0	5.7	6.9	7.5	<xsn								
STIB 386	0	5.7	6.9	7.5	<xsn									
STIB 386	0	0	6.9	7.5	<xsn									
STIB 386	0	0	0	0	0	0	0	0	0	0	0	0	0	0
STIB 386	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2 mg/kg														
STIB 386	0	0	0	0	0	0	0	0	0	0	0	0	0	0
STIB 386	0	0	0	5.7	6.9	7.2	8.1	<xsn						
STIB 386	0	0	0	0	0	0	0	0	0	0	0	0	0	0
STIB 386	0	0	0	0	0	0	0	0	0	0	0	0	0	0
STIB 386	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5 mg/kg														
STIB 386	0	0	0	0	0	0	0	0	0	0	0	0	0	0
STIB 386	0	0	0	0	0	0	0	0	0	0	0	0	0	0
STIB 386	0	0	0	0	0	0	0	0	0	0	0	0	0	0
STIB 386	0	0	0	0	0	0	0	0	0	0	0	0	0	0
STIB 386	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CONTROL														
STIB 386	0	5.7	6.9	7.5	<xsn									
STIB 386	0	0	6.9	7.2	7.5	<xsn								

Table A3. A Cont: *In vivo* screening of the three parental lines for Cymelarsan resistance

Dose MelCy						PARASITAEMIA								
0.2 mg/kg	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
TREU 927	0	5.7	7.2	8.1	<xsn									
TREU 927	0	0	5.7	6.9	7.5	<xsn								
TREU 927	0	5.7	6.9	7.5	<xsn									
TREU 927	0	0	6.9	7.5	<xsn									
TREU 927	0	6.9	7.5	<xsn										
0.5 mg/kg														
TREU 927	0	0	5.7	6.9	7.5	<xsn								
TREU 927	0	5.7	7.2	7.5	<xsn									
TREU 927	0	0	5.7	6.9	7.5	<xsn								
TREU 927	0	6.9	7.2	8.1	<xsn									
TREU 927	0	5.7	6.9	7.5	<xsn									
1 mg/kg														
TREU 927	0	0	0	0	5.7	6.9	7.2							
TREU 927	0	0	0	5.7	7.2	8.1	<xsn							
TREU 927	0	0	6.9	7.5	<xsn									
TREU 927	0	5.7	6.9	7.2	7.5	<xsn								
TREU 927	0	0	6.9	7.2	8.1	<xsn								
2 mg/kg														
TREU 927	0	0	0	5.7	6.9	7.5	<xsn							
TREU 927	0	0	6.9	7.2	8.1	<xsn								
TREU 927	0	0	5.7	6.9	7.5	<xsn								
TREU 927	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TREU 927	0	5.7	6.9	7.2	7.5	<xsn								
5 mg/kg														
TREU 927	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TREU 927	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TREU 927	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TREU 927	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TREU 927	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CONTROL														
TREU 927	0	0	6.9	7.5	<xsn									
TREU 927	0	5.7	6.9	7.2	8.1	<xsn								

Table A3.A: Screening of the drug resistance phenotype of the three parental lines, *in vivo*, with increasing doses of Cymelarsan (MelCy). The tables indicate the results for the mouse screens used to determine the resistance phenotype of the three parental lines. The minimum inhibitory concentration (MIC) of MelCy required to clear infection of STIB 247 was 0.5 mg/kg. The MIC of MelCy required to clear STIB 386 and TREU 927 infection was 5 mg/kg. These results indicated that STIB 386 and TREU 927 displayed a resistance phenotype in comparison to STIB 247. Number trypanosomes/ml = anti log parasitaemia value (Lumsden *et al*, 1963).

Table A3.B: *In vivo* screening of six F1 hybrid progeny for their Cymelarsan resistance phenotype from the STIB 247 x TREU 927 cross

Dose MelCy	PARASITAEMIA													
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
0.1 mg/kg														
F532/72 MCL 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/72 MCL 2	0	0	5.7	6.9	7.5	<xsn								
F532/72 MCL 2	0	0	5.7	6.9	7.5	<xsn								
F532/72 MCL 2	0	0	5.7	7.2	<xsn									
F532/72 MCL 2	0	0	0	5.7	7.2	<xsn								
0.2 mg/kg														
F532/72 MCL 2	0	0	5.7	6.9	7.2	7.5	<xsn							
F532/72 MCL 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/72 MCL 2	0	0	0	5.7	7.2	<xsn								
F532/72 MCL 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/72 MCL 2	0	0	5.7	6.9	7.5	<xsn								
0.5 mg/kg														
F532/72 MCL 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/72 MCL 2	0	0	0	0	0	6.9	7.2	<xsn						
F532/72 MCL 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/72 MCL 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/72 MCL 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1 mg/kg														
F532/72 MCL 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/72 MCL 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/72 MCL 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/72 MCL 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/72 MCL 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2 mg/kg														
F532/72 MCL 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/72 MCL 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/72 MCL 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/72 MCL 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/72 MCL 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CONTROL														
F532/72 MCL 2	0	6.9	7.2	8.1	<xsn									
F532/72 MCL 2	0	5.7	6.9	7.5	<xsn									

Table A3.B Cont: *In vivo* screening of six F1 hybrid progeny for their Cymelarsan resistance phenotype from the STIB 247 x TREU 927 cross

Dose MelCy							PARASITAEMIA							
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
0.1 mg/kg														
F124/28 BCL20	0	5.7	6.9	7.5	<xsn									
F124/28 BCL20	0	0	5.7	6.9	7.5	<xsn								
F124/28 BCL20	0	0	5.7	6.9	7.2	<xsn								
F124/28 BCL20	0	0	0	0	0	0	0	o	o	o	o	o	o	o
F124/28 BCL20	0	0	0	6.9	7.5	<xsn								
0.2 mg/kg														
F124/28 BCL20	0	0	0	5.7	6.9	7.2	<xsn							
F124/28 BCL20	0	0	0	0	0	0	0	o	o	o	o	o	o	o
F124/28 BCL20	0	0	5.7	6.9	7.5	<xsn								
F124/28 BCL20	0	0	0	0	0	0	0	o	o	o	o	o	o	o
F124/28 BCL20	0	0	0	6.9	7.2	8.1	<xsn							
0.5 mg/kg														
F124/28 BCL20	0	0	0	0	0	0	0	o	o	o	o	o	o	o
F124/28 BCL20	0	0	0	0	0	0	0	o	o	o	o	o	o	o
F124/28 BCL20	0	0	0	0	0	0	0	o	o	o	o	o	o	o
F124/28 BCL20	0	0	0	0	0	0	0	o	o	o	o	o	o	o
F124/28 BCL20	0	0	0	0	0	0	0	o	o	o	o	o	o	o
1 mg/kg														
F124/28 BCL20	0	0	0	0	0	0	0	o	o	o	o	o	o	o
F124/28 BCL20	0	0	0	0	0	0	0	o	o	o	o	o	o	o
F124/28 BCL20	0	0	0	0	0	0	0	o	o	o	o	o	o	o
F124/28 BCL20	0	0	0	0	0	0	0	o	o	o	o	o	o	o
F124/28 BCL20	0	0	0	0	0	0	0	o	o	o	o	o	o	o
2 mg/kg														
F124/28 BCL20	0	0	0	0	0	0	0	o	o	o	o	o	o	o
F124/28 BCL20	0	0	0	0	0	0	0	o	o	o	o	o	o	o
F124/28 BCL20	0	0	0	0	0	0	0	o	o	o	o	o	o	o
F124/28 BCL20	0	0	0	0	0	0	0	o	o	o	o	o	o	o
F124/28 BCL20	0	0	0	0	0	0	0	o	o	o	o	o	o	o
CONTROL														
F124/28 BCL20	0	0	5.7	7.2	8.1	<xsn								
F124/28 BCL20	0	0	0	6.9	7.5	<xsn								

Table A3.B Cont: *In vivo screening of six F1 hybrid progeny for their Cymelarsan resistance phenotype from the STIB 247 x TREU 927 cross*

Dose MelCy	PARASITAEMIA													
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
0.1 mg/kg														
F532/72 BCL 1	0	0	5.7	7.2	7.5	<xsn								
F532/72 BCL 1	0	5.7	6.9	7.5	<xsn									
F532/72 BCL 1	0	0	8.9	7.2	7.5	<xsn								
F532/72 BCL 1	0	0	5.7	6.9	7.2	7.5	<xsn							
F532/72 BCL 1	0	0	0	5.7	6.9	7.5	<xsn							
0.2 mg/kg														
F532/72 BCL 1	0	0	0	0	6.9	7.2	7.5	<xsn						
F532/72 BCL 1	0	0	0	5.7	6.9	7.5	<xsn							
F532/72 BCL 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/72 BCL 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/72 BCL 1	0	0	5.7	7.2	7.5	<xsn								
0.5 mg/kg														
F532/72 BCL 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/72 BCL 1	0	0	0	5.7	7.2	7.5	<xsn							
F532/72 BCL 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/72 BCL 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/72 BCL 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1 mg/kg														
F532/72 BCL 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/72 BCL 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/72 BCL 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/72 BCL 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/72 BCL 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2 mg/kg														
F532/72 BCL 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/72 BCL 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/72 BCL 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/72 BCL 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/72 BCL 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CONTROL														
F532/72 BCL 1	5.7	6.9	7.5	<xsn										
F532/72 BCL 1	0	5.7	7.2	8.1	<xsn									

Table A3.B Cont: *In vivo* screening of six F1 hybrid progeny for their Cymelarsan resistance phenotype from the STIB 247 x TREU 927 cross

Dose MelCy	PARASITAEMIA													
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
0.2 mg/kg														
F532/63 BCL 2	0	0	5.7	6.9	7.2	7.5	<xsn							
F532/63 BCL 2	0	5.7	6.9	7.5	<xsn									
F532/63 BCL 2	0	0	0	5.7	7.2	<xsn								
F532/63 BCL 2	0	0	0	5.7	6.9	7.5	<xsn							
F532/63 BCL 2	0	0	5.7	6.9	7.2	8.1	<xsn							
0.5 mg/kg														
F532/63 BCL 2	0	0	0	5.7	7.2	8.1	<xsn							
F532/63 BCL 2	0	0	0	0	5.7	6.9	7.2	<xsn						
F532/63 BCL 2	0	0	6.9	7.2	8.1	<xsn								
F532/63 BCL 2	0	0	0	6.9	7.5	<xsn								
F532/63 BCL 2	0	0	5.7	7.2	7.5	<xsn								
1 mg/kg														
F532/63 BCL 2	0	0	6.9	7.5	<xsn									
F532/63 BCL 2	0	0	0	5.7	7.2	7.5	<xsn							
F532/63 BCL 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/63 BCL 2	0	0	5.7	7.2	7.5	<xsn								
F532/63 BCL 2	0	0	0	0	6.9	7.5	<xsn							
2 mg/kg														
F532/63 BCL 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/63 BCL 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/63 BCL 2	0	0	5.7	5.7	6.9	7.5	<xsn							
F532/63 BCL 2	0	0	0	0	5.7	7.2	7.5	<xsn						
F532/63 BCL 2	0	0	0	0	6.9	7.2	7.5	<xsn						
5 mg/kg														
F532/63 BCL 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/63 BCL 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/63 BCL 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/63 BCL 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F532/63 BCL 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CONTROL														
F532/63 BCL 2	0	0	0	6.9	7.5	8.1	<xsn							
F532/63 BCL 2	0	0	5.7	7.2	7.5	<xsn								

Table A3.B Cont: *In vivo* screening of six F1 hybrid progeny for their Cymelarsan resistance phenotype from the STIB 247 x TREU 927 cross

Dose MelCy		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
0.2 mg/kg															
F532/63CL A11		0	0	5.7	7.2	8.1	<xsn								
F532/63CL A11		0	0	6.9	7.2	8.1	<xsn								
F532/63CL A11		0	0	5.7	6.9	7.5	<xsn								
F532/63CL A11		0	0	0	0	5.7	7.2	8.1	<xsn						
F532/63CL A11		0	0	0	5.7	7.2	8.1	<xsn							
0.5 mg/kg															
F532/63CL A11		0	0	0	5.7	6.9	7.2	<xsn							
F532/63CL A11		0	0	0	0	6.9	7.5	<xsn							
F532/63CL A11		0	0	5.7	6.9	7.5	<xsn								
F532/63CL A11		0	0	0	0	6.9	7.5	<xsn							
F532/63CL A11		0	0	0	6.9	7.2	8.1	<xsn							
1 mg/kg															
F532/63CL A11		0	0	5.7	7.2	7.5	<xsn								
F532/63CL A11		0	0	0	0	0	0	0	o	o	o	o	o	o	o
F532/63CL A11		0	0	0	6.9	7.5	<xsn								
F532/63CL A11		0	5.7	6.9	7.5	<xsn									
F532/63CL A11		0	0	6.9	7.2	8.1	<xsn								
2 mg/kg															
F532/63CL A11		0	0	0	0	0	0	0	o	o	o	o	o	o	o
F532/63CL A11		0	0	0	0	0	0	0	o	o	o	o	o	o	o
F532/63CL A11		0	0	5.7	6.9	7.5	<xsn								
F532/63CL A11		0	0	0	6.9	7.2	8.1	<xsn							
F532/63CL A11		0	0	0	0	0	0	0	o	o	o	o	o	o	o
5 mg/kg															
F532/63CL A11		0	0	0	0	0	0	0	o	o	o	o	o	o	o
F532/63CL A11		0	0	0	0	0	0	0	o	o	o	o	o	o	o
F532/63CL A11		0	0	0	0	0	0	0	o	o	o	o	o	o	o
F532/63CL A11		0	0	0	0	0	0	0	o	o	o	o	o	o	o
F532/63CL A11		0	0	0	0	0	0	0	o	o	o	o	o	o	o
CONTROL															
F532/63CL A11		0	5.7	6.9	7.5	<xsn									
F532/63CL A11		0	0	6.9	7.5	<xsn									

PARASITAEMIA

Table A3.B Cont: *In vivo* screening of six F1 hybrid progeny for their Cymelarsan resistance phenotype from the STIB 247 x TREU 927 cross

Dose MelCy	PARASITAEMIA													
0.2 mg/kg	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
F974/78 BCL 3	0	0	6.9	7.2	7.5	<xsn								
F974/78 BCL 3	0	0	5.7	7.2	7.5	<xsn								
F974/78 BCL 3	0	5.7	6.9	7.5	<xsn									
F974/78 BCL 3	0	0	5.7	7.2	8.1	<xsn								
F974/78 BCL 3	0	0	0	5.7	6.9	7.2	<xsn							
0.5 mg/kg														
F974/78 BCL 3	0	5.7	6.9	7.5	<xsn									
F974/78 BCL 3	0	0	0	6.9	7.5	<xsn								
F974/78 BCL 3	0	0	6.9	7.2	8.1	<xsn								
F974/78 BCL 3	0	0	5.7	7.2	7.5	<xsn								
F974/78 BCL 3	0	5.7	6.9	7.2	<xsn	<xsn								
1 mg/kg														
F974/78 BCL 3	0	0	0	6.9	7.2	7.5	<xsn							
F974/78 BCL 3	0	0	5.7	6.9	7.5	<xsn								
F974/78 BCL 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F974/78 BCL 3	0	5.7	6.9	7.5	<xsn									
F974/78 BCL 3	0	0	6.9	7.2	7.5	<xsn								
2 mg/kg														
F974/78 BCL 3	0	5.7	6.9	7.2	7.5	<xsn								
F974/78 BCL 3	0	0	6.9	7.5	<xsn									
F974/78 BCL 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F974/78 BCL 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F974/78 BCL 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5 mg/kg														
F974/78 BCL 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F974/78 BCL 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F974/78 BCL 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F974/78 BCL 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F974/78 BCL 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CONTROL														
F974/78 BCL 3	0	5.7	7.2	7.5	<xsn									
F974/78 BCL 3	0	0	6.9	7.2	7.5	<xsn								

Table A3.B: Screening of the drug resistance phenotype of six F1 hybrids from the STIB 247 x TREU 927 cross, in vivo, with increasing doses of Cymelarsan (MelCy). The tables indicate the results for the mouse screens used to determine the resistance phenotype of the six F1 hybrids. The minimum inhibitory concentration (MIC) of MelCy required to clear infection of F532/72 MCL 2, F124/28 BCL 20 and F532/72 BCL 1 was 0.5 mg/kg and so had sensitive phenotypes. The MIC of MelCy required to clear F532/63 BCL 2, F532 CL A11 and F974/78 BCL 3 infections was 5 mg/kg and so had resistant phenotypes. Number trypanosomes/ml = anti log parasitaemia value (Lumsden et al, 1963).

Table A3.C: *In vivo* screening of six F1 hybrid progeny for their Cymelarsan resistance phenotype from the STIB 247 x STIB 386 cross

Dose MelCy	PARASITAEMIA													
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
0.1 mg/kg														
F9/45 MCL 11	0	0	6.9	7.2	7.5	<xsn								
F9/45 MCL 11	0	5.7	7.2	7.5	<xsn									
F9/45 MCL 11	0	0	0	6.9	7.2	7.5	<xsn							
F9/45 MCL 11	0	0	6.9	7.5	<xsn									
F9/45 MCL 11	0	0	5.7	7.2	7.5	<xsn								
0.2 mg/kg														
F9/45 MCL 11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F9/45 MCL 11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F9/45 MCL 11	0	5.7	7.2	8.1	<xsn									
F9/45 MCL 11	0	0	5.7	6.6	7.5	<xsn								
F9/45 MCL 11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.5 mg/kg														
F9/45 MCL 11	0	0	0	5.7	7.2	7.5	<xsn							
F9/45 MCL 11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F9/45 MCL 11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F9/45 MCL 11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1 mg/kg														
F9/45 MCL 11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F9/45 MCL 11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F9/45 MCL 11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F9/45 MCL 11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F9/45 MCL 11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2 mg/kg														
F9/45 MCL 11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F9/45 MCL 11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F9/45 MCL 11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F9/45 MCL 11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F9/45 MCL 11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CONTROL														
F9/45 MCL 11	0	5.7	6.9	7.5	<xsn									
F9/45 MCL 11	0	0	5.7	7.2	8.1	<xsn								

Table A3.C Cont: *In vivo* screening of six F1 hybrid progeny for their Cymelarsan resistance phenotype from the STIB 247 x STIB 386 cross

Dose MelCy	PARASITAEMIA													
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
0.1 mg/kg														
F492/50 BCL23	0	0	5.7	7.2	7.5	<xsn								
F492/50 BCL23	0	0	5.7	6.9	7.5	<xsn								
F492/50 BCL23	0	5.7	6.9	7.2	7.5	<xsn								
F492/50 BCL23	0	0	0	6.9	7.2	7.5	<xsn							
F492/50 BCL23	0	5.7	7.2	7.5	<xsn									
0.2 mg/kg														
F492/50 BCL23	0	0	0	0	0	0	0	o	o	o	o	o	o	o
F492/50 BCL23	0	0	6.9	7.5	<xsn									
F492/50 BCL23	0	5.7	6.9	7.2	7.5	<xsn								
F492/50 BCL23	0	0	0	0	0	0	0	o	o	o	o	o	o	o
F492/50 BCL23	0	0	0	5.7	6.9	7.5	<xsn							
0.5 mg/kg														
F492/50 BCL23	0	0	0	0	0	0	o	o	o	o	o	o	o	o
F492/50 BCL23	0	0	0	0	0	0	o	o	o	o	o	o	o	o
F492/50 BCL23	0	0	0	0	0	0	o	o	o	o	o	o	o	o
F492/50 BCL23	0	0	0	0	0	0	o	o	o	o	o	o	o	o
1 mg/kg														
F492/50 BCL23	0	0	0	0	0	0	o	o	o	o	o	o	o	o
F492/50 BCL23	0	0	0	0	0	0	o	o	o	o	o	o	o	o
F492/50 BCL23	0	0	0	0	0	0	o	o	o	o	o	o	o	o
F492/50 BCL23	0	0	0	0	0	0	o	o	o	o	o	o	o	o
F492/50 BCL23	0	0	0	0	0	0	o	o	o	o	o	o	o	o
2 mg/kg														
F492/50 BCL23	0	0	0	0	0	0	o	o	o	o	o	o	o	o
F492/50 BCL23	0	0	0	0	0	0	o	o	o	o	o	o	o	o
F492/50 BCL23	0	0	0	0	0	0	o	o	o	o	o	o	o	o
F492/50 BCL23	0	0	0	0	0	0	o	o	o	o	o	o	o	o
F492/50 BCL23	0	0	0	0	0	0	o	o	o	o	o	o	o	o
CONTROL														
F492/50 BCL23	0	0	6.9	7.5	<xsn									
F492/50 BCL23	0	5.7	6.9	7.5	<xsn									

Table A3.C Cont: *In vivo* screening of six F1 hybrid progeny for their Cymelarsan resistance phenotype from the STIB 247 x STIB 386 cross

Dose MelCy	PARASITAEMIA													
0.1 mg/kg	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
F19/31 BCL11	0	0	6.9	7.5	<xsn	<xsn								
F19/31 BCL11	0	5.7	6.9	7.2	7.5	<xsn								
F19/31 BCL11	0	0	5.7	7.2	7.5	<xsn								
F19/31 BCL11	0	0	6.9	7.2	7.5	<xsn								
F19/31 BCL11	0	5.7	7.2	7.5	<xsn	<xsn								
0.2 mg/kg														
F19/31 BCL11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F19/31 BCL11	0	0	5.7	6.9	7.5	<xsn								
F19/31 BCL11	0	6.9	7.2	7.5	<xsn									
F19/31 BCL11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F19/31 BCL11	0	0	5.7	6.9	7.5	<xsn								
0.5 mg/kg														
F19/31 BCL11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F19/31 BCL11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F19/31 BCL11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F19/31 BCL11	0	0	0	0	5.7	6.9	7.5	<xsn						
F19/31 BCL11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1 mg/kg														
F19/31 BCL11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F19/31 BCL11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F19/31 BCL11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F19/31 BCL11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F19/31 BCL11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2 mg/kg														
F19/31 BCL11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F19/31 BCL11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F19/31 BCL11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F19/31 BCL11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F19/31 BCL11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CONTROL														
F19/31 BCL11	0	0	5.7	6.9	7.5	<xsn								
F19/31 BCL11	0	5.7	7.2	7.5	<xsn									

Table A3.C Cont: *In vivo* screening of six F1 hybrid progeny for their Cymelarsan resistance phenotype from the STIB 247 x STIB 386 cross

Dose MelCy	PARASITAEMIA													
0.2 mg/kg	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
F492/50 BCL 8	0	6.9	7.2	7.5	<xsn									
F492/50 BCL 8	0	5.7	7.2	7.5	<xsn									
F492/50 BCL 8	0	0	6.9	7.5	<xsn									
F492/50 BCL 8	0	6.9	7.5	<xsn										
F492/50 BCL 8	0	6.9	7.5	<xsn										
0.5 mg/kg														
F492/50 BCL 8	0	5.7	7.2	<xsn										
F492/50 BCL 8	0	6.9	7.5	<xsn										
F492/50 BCL 8	0	0	5.7	6.9	7.5	<xsn								
F492/50 BCL 8	0	5.7	6.9	7.5	<xsn									
F492/50 BCL 8	0	0	6.9	7.5	<xsn									
1 mg/kg														
F492/50 BCL 8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F492/50 BCL 8	0	6.9	7.5	<xsn										
F492/50 BCL 8	0	0	0	0	5.7	7.2	8.1	<xsn						
F492/50 BCL 8	0	5.7	6.9	7.5	<xsn									
F492/50 BCL 8	0	0	0	5.7	6.9	7.5	<xsn							
2 mg/kg														
F492/50 BCL 8	0	0	0	0	6.9	7.2	7.5	<xsn						
F492/50 BCL 8	0	0	5.7	6.9	7.5	<xsn								
F492/50 BCL 8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F492/50 BCL 8	0	0	0	6.9	7.5	<xsn								
F492/50 BCL 8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5 mg/kg														
F492/50 BCL 8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F492/50 BCL 8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F492/50 BCL 8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F492/50 BCL 8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F492/50 BCL 8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CONTROL														
F492/50 BCL 8	0	0	6.9	7.5	<xsn									
F492/50 BCL 8	0	5.7	7.2	7.5	<xsn									

Table A3.C Cont: *In vivo* screening of six F1 hybrid progeny for their Cymelarsan resistance phenotype from the STIB 247 x STIB 386 cross

Dose MelCy	PARASITAEMIA													
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
0.2 mg/kg														
F492/50 BCL21	0	5.7	6.9	7.2	7.5	<xsn								
F492/50 BCL21	0	5.7	7.2	7.5	<xsn									
F492/50 BCL21	0	0	6.9	7.5	<xsn									
F492/50 BCL21	0	5.7	7.2	7.5	<xsn									
F492/50 BCL21	0	0	5.7	6.9	7.5	<xsn								
0.5 mg/kg														
F492/50 BCL21	0	0	0	6.9	7.2	8.1	<xsn							
F492/50 BCL21	0	5.7	6.9	7.5	<xsn									
F492/50 BCL21	0	0	5.7	6.9	7.5	<xsn								
F492/50 BCL21	0	0	5.7	7.2	8.1	<xsn								
F492/50 BCL21	0	0	0	6.9	7.5	<xsn								
1 mg/kg														
F492/50 BCL21	0	0	0	0	0	0	0	o	o	o	o	o	o	o
F492/50 BCL21	0	0	0	5.7	7.2	8.1	<xsn							
F492/50 BCL21	0	0	5.7	7.2	8.1	<xsn								
F492/50 BCL21	0	0	0	5.7	6.9	7.5	<xsn							
F492/50 BCL21	0	0	5.7	6.9	7.5	<xsn								
2 mg/kg														
F492/50 BCL21	0	0	0	0	0	0	0	o	o	o	o	o	o	o
F492/50 BCL21	0	0	0	5.7	6.9	7.5	<xsn							
F492/50 BCL21	0	0	0	0	0	0	0	o	o	o	o	o	o	o
F492/50 BCL21	0	0	0	0	0	0	0	o	o	o	o	o	o	o
F492/50 BCL21	0	0	0	0	0	0	0	o	o	o	o	o	o	o
5 mg/kg														
F492/50 BCL21	0	0	0	0	0	0	0	o	o	o	o	o	o	o
F492/50 BCL21	0	0	0	0	0	0	0	o	o	o	o	o	o	o
F492/50 BCL21	0	0	0	0	0	0	0	o	o	o	o	o	o	o
F492/50 BCL21	0	0	0	0	0	0	0	o	o	o	o	o	o	o
F492/50 BCL21	0	0	0	0	0	0	0	o	o	o	o	o	o	o
CONTROL														
F492/50 BCL21	0	5.7	6.9	7.5	<xsn									
F492/50 BCL21	0	0	5.7	7.2	8.1	<xsn								

Table A3.C Cont: *In vivo* screening of six F1 hybrid progeny for their Cymelarsan resistance phenotype from the STIB 247 x STIB 386 cross

Dose MelCy	PARASITAEMIA													
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
0.2 mg/kg														
F9/41 BCL 7	0	5.7	6.9	7.5	<xsn									
F9/41 BCL 7	0	5.7	6.9	7.2	7.5	<xsn								
F9/41 BCL 7	0	0	6.9	7.2	8.1	<xsn								
F9/41 BCL 7	0	5.7	6.9	7.5	<xsn									
F9/41 BCL 7	0	0	6.8	7.5	<xsn									
0.5 mg/kg														
F9/41 BCL 7	0	0	5.7	7.2	7.5	<xsn								
F9/41 BCL 7	0	5.7	6.9	7.5	<xsn									
F9/41 BCL 7	0	5.7	7.2	8.1	<xsn									
F9/41 BCL 7	0	0	6.9	7.5	<xsn									
F9/41 BCL 7	0	0	0	5.7	6.9	7.5	<xsn							
1 mg/kg														
F9/41 BCL 7	0	0	0	0	0	0	0	0	0	0	0	0	0	
F9/41 BCL 7	0	5.7	6.9	7.5	<xsn									
F9/41 BCL 7	0	0	5.7	7.2	8.1	<xsn								
F9/41 BCL 7	0	0	0	0	0	0	0	0	0	0	0	0	0	
F9/41 BCL 7	0	0	5.7	6.9	7.2	7.5	<xsn							
2 mg/kg														
F9/41 BCL 7	0	0	5.7	7.2	8.1	<xsn								
F9/41 BCL 7	0	0	0	0	0	0	0	0	0	0	0	0	0	
F9/41 BCL 7	0	5.7	6.9	7.2	7.5	<xsn								
F9/41 BCL 7	0	0	0	0	0	0	0	0	0	0	0	0	0	
F9/41 BCL 7	0	0	0	0	0	0	0	0	0	0	0	0	0	
5 mg/kg														
F9/41 BCL 7	0	0	0	0	0	0	0	0	0	0	0	0	0	
F9/41 BCL 7	0	0	0	0	0	0	0	0	0	0	0	0	0	
F9/41 BCL 7	0	0	0	0	0	0	0	0	0	0	0	0	0	
F9/41 BCL 7	0	0	0	0	0	0	0	0	0	0	0	0	0	
F9/41 BCL 7	0	0	0	0	0	0	0	0	0	0	0	0	0	
CONTROL														
F9/41 BCL 7	0	0	6.9	7.5	<xsn									
F9/41 BCL 7	0	5.7	6.9	7.5	<xsn									

Table A3.C: Screening of the drug resistance phenotype of six F1 hybrids from the STIB 247 x STIB 386 cross, *in vivo*, with increasing doses of Cymelarsan (MelCy). The tables indicate the results for the mouse screens used to determine the resistance phenotype of the six F1 hybrids. The minimum inhibitory concentration (MIC) of MelCy required to clear infection of F9/45 MCL 11, F492/50 BCL 23 and F19/31 BCL 11 was 0.5 mg/kg and so had sensitive phenotypes. The MIC of MelCy required to clear F492/50 BCL 8, F492/50 BCL 21 and F9/41 BCL 7 infections was 5 mg/kg and so had resistant phenotypes. Number trypanosomes/ml = anti log parasitaemia value (Lumsden *et al*, 1963).

REFERENCES

Bacchi, C.J. and Yarlett, N. (1993). Effects of antagonists of polyamine metabolism on African trypanosomes. *Acta Tropica* **54,** 225-236.

Bakker, B.M., Westerhoff, H.V., Opperdoes, F.R. and Michels, P.A.M. (2000). Metabolic control analysis of glycolysis in trypanosomes as an approach to improve selectivity and effectiveness of drugs. *Molecular and Biochemical Parasitology* **106,** 1-10.

Barrett, M.P., MacLeod, A., Tovar, J., Sweetman, J.P., Tait, A., LePage, R.W.F. and Melville, S.E. (1997). A single locus minisatellite sequence which distinguishes between *Trypanosoma brucei* isolates. *Molecular and Biochemical Parasitology* **86,** 95-99.

Barrett, M.P. and Fairlamb, A.H. (1999). The biochemical basis of arsenical-diamidine cross-resistance in African trypanosomes. *Parasitology Today* **15,** 136-140.

Barrett, M.P., Burchmore, R.J.S., Stich, A., Lazzari, J.O., Frasch, A.C. and Cazzulo, J.J. (2003). The trypanosomiases. *The Lancet* **362,** 1469-1480.

Bastin, P., Bagherzadeh, A., Matthews, K.R. and Gull, K. (1996). A novel epitope tag system to study protein targeting and organelle biogenesis in *Trypanosoma brucei. Molecular and Biochemical Parasitology* **77,** 235-239.

Bastin, P., MacRae, T.H., Francis, S.B., Matthews, M.R. and Gull, K. (1999). Flagellar morphogenesis: Protein targeting and assembly in the paraflagellar rod of trypanosomes. *Molecular and Cell Biology,* 8191-8200.

Benson, G. (1999). Tandem repeats finder: a program to analyse DNA sequences. *Nucleic Acids Research* **27**, 573-580.

Bentivoglio, M., Grassi-Zucconi, G., Olsson, T. and Kristensson, K. (1994). *Trypanosoma brucei* and the nervous system. *Trends in Neuroscience* **17**, 325-329.

Bourn, D., Reid, R., Rogers, D., Snow, B. and Wint, W. (2001) Environmental Change and the Autonomous Control of Tsetse and Trypanosomiasis in Sub-Saharan Africa. *Environmental Research Group Oxford Limited.*

Brown, R.C., Evans, D.A. and Vickerman, K. (1973). Changes in oxidative metabolism and ultrastructure accompanying differentiation of the mitochondrion in *Trypanosoma brucei. International Journal for Parasitology* **3**, 691-704.

Brun, R. and Schonenberger, M. (1979). Cultivation and *in vitro* cloning of procyclic culture forms of *Trypanosoma brucei* in a semi-defined medium. Short communication. *Acta Tropica* **36**, 289-292.

Brun, R. and Jenni, L. (1985). Cultivation of African and South American Trypanosomes of medical or veterinary importance. *British Medical Bulletin* **41**, 122-129.

Brun, R.,Baeriswyl, S. and Kunz, C. (1989). In vitro drug sensitivity of *Trypanosoma gambiense* isolates. *Acta Tropica* **46**, 369-376.

Burri, C., Baltz, T., Giroud, C., Doua, F., Welker, H.A. and Brun, R. (1993). Pharmacokinetic properties of the trypanocidal drug melarsoprol. *Chemotherapy* **39**, 225-234.

Burri, C., Nkunku, S., Merolle, A., Smith, T., Blum, J. and Brun, R. (2000). Efficacy of a new, concise schedule for melarsoprol in treatment of sleeping caused by *Trypanosoma brucei gambiense*: a randomised trial. *The Lancet* **355**, 1419-1425.

Burri, C., Yeramian, P.D., Allen, J.L., Tidwell, R.R., Merolle, A., Kyanza, S.K., Tshindele, P.L., Bilenge, C.M.M., Manuel, F., Josenando, T. and Pohlig, G. (2003). Efficacy and safety of DB289, a new oral drug for treatment of first stage sleeping sickness: an open-label, non-controlled phase IIA trial. *American Society of Tropical Medicine and Hygiene Meeting*, Philadelphia, USA.

Carter, N.S. and Fairlamb, A.H. (1993). Arsenical-resistant trypanosomes lack an unusual adenosine transporter. *Nature (Lond)* **361**, 173-175.

Carter, N.S., Barrett, M.P. and de Koning, H.P. (1999). A drug resistance determinant in *Trypanosoma brucei*. *Trends in Microbiology* **7**, 469-471.

Clayton, C.E. (1987). Import of fructose biphosphate aldolase into the glycosomes of *Trypanosoma brucei*. *Journal of Cell Biology* **105**, 2649-2654.

Cross, G.A.M. (1975). Identification, purification and properties of clone-specific glycoprotein antigens constituting the surface coat of *Trypanosoma brucei*. *Parasitology* **71**, 393-417.

Cunningham, I. (1977). New culture medium for maintenance of tsetse tissues and growth of trypanosomatids. *Journal of Protozoology* **24**, 325-329.

de Koning, H.P. and Jarvis, S.M. (1997). Purine nucleobase transport in bloodstream forms of *Trypanosoma brucei brucei* is mediated by two novel transporters. *Molecular and Biochemical Parasitology* **89**, 245-258.

de Koning, H.P and Jarvis, S.M. (1998). A highly selective, high-affinity transporter for uracil in *Trypanosoma brucei brucei*; evidence for proton-dependent transport. *Biochemical Cell Biology* **76**, 853-858.

de Koning, H.P. and Jarvis, S.M. (1999). Adenosine transporters in bloodstream forms of *Trypanosoma brucei brucei*: substrate recognition motifs and affinity for trypanocidal drugs. *Molecular Pharmacology* **56**, 1162-1170.

de Koning, H.P., MacLeod, A., Barrett, M.P., Cover, B. and Jarvis, S.M. (2000). Further evidence for a link between melarsoprol resistance and P2 transporter function in African trypanosomes. *Molecular and Biochemical Parasitology* **106**, 181-185.

de Koning, H.P. (2001A). Uptake of pentamidine in *Trypanosoma brucei brucei* is mediated by three distinct transporters: Implications for cross-resistance with arsenicals. *Molecular Pharmacology* **59**, 586-592.

de Koning, H.P. (2001B). Transporters in African trypanosomes: role in drug action and resistance. *International Journal for Parasitology* **31**, 512-522.

de Koning, H.P. and Jarvis, S.M. (2001). Uptake of pentamidine in *Trypanosoma brucei brucei* is mediated by the P2 adenosine transporter and at least one novel, unrelated transporter. *Acta Tropica* **80**, 245-250.

Denise, H. and Barrett, M.P. (2001). Uptake and mode of action of drugs used against sleeping sickness. *Biochemical Pharmacology* **60**, 1-5.

Denise, H., Giroud, C., Barrett, M.P. and Baltz, T. (1999). Affinity chromatography using trypanocidal arsenical drugs identifies a specific interaction between glycerol-3-phosphate dehydrogenase from *Trypanosoma brucei* and Cymelarsan. *European Journal of Biochemistry* **259**, 339-346.

Djimde, A., Doumbo, O.K., Cortese, J.K., Kayentao, K., Doumbo, S., Diourte, Y., Dicko, A., Su, X.Z., Nomura, T., Fidock, D.A., Wellems, T.E. and Plowe, C.V. (2001). A molecular marker for chloroquine-resistant falciparum malaria. *New England Journal of Medicine* **344**, 257-263.

Duan, J., Wainwright, M.S., Comeron, J.M., Saitou, N., Sanders, A.R., Gelernter, J. and Gejman, P.V. (2003). Synonymous mutations in the human dopamine receptor D2 (DRD2) affect mRNA stability and synthesis of the receptor. *Human Molecular Genetics* **12**, 205-216.

El-Sayed, N.M.A., Ghedin, E., Song, J., MacLeod, A., Bringaud, F., Larkin, C., Wanless, D., Peterson, J., Hou, L., Taylor, S., Tweedie, A., Biteau, N., Khalak, H.G., Lin, X., Mason, T., Hannick, L., Caler, E., Blandin, G., Bartholomeu, D., Simpson, A.J., Kaul, S., Zhao, H., Pai, G., Van Aken, S., Utterback, T., Haas, B., Koo, H.L., Umayam, L., Suh, B., Gerrard, C., Leech, V., Qi, R., Zhou, S., Schwartz, D., Feldblyum, T., Salzberg, S., Tait, A., Turner, C.M.R., Ullu, E., White, O., Melville, S., Adams, M.D., Fraser, C.M. and Donelson, J.E. (2003). The sequence and analysis of *Trypanosoma brucei* chromosome II. *Nucleic Acids Research* **31**, 4856-4863.

Fairlamb, A.H., Henderson, G.B. and Cerami, A. (1989). Trypanothione is the primary target for arsenical drugs against African trypanosomes. *Proceedings of the National Academy of Sciences*, USA **86**, 2607-2611.

Fairlamb, A.H., Carter, N.S., Cunningham, M. and Smith, K. (1992). Characterisation of melarsen-resistant *Trypanosoma brucei brucei* with respect to cross-resistance to other drugs and trypanothione metabolism. *Molecular and Biochemical Parasitology* **53**, 213-222.

Ferdig, M.T. and Su, X-z. (2000). Microsatellite markers and genetic mapping in *Plasmodium falciparum*. *Parasitology Today* **16**, 307-312.

Fidock, D.A., Nomura, T., Talley, A.K., Cooper, R.A., Dzekunov, S.M., Ferdig, M.T., Ursos, L.M.B., Sidhu, A.B.S., Naude, B., Deitsch, K.W., Su, X., Wootton, J.C., Roepe, P.D. and Wellems, T.E. (2000). Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in Chloroquine resistance. *Molecular Cell* **6**, 861-871.

Gaud, A., Carrington, M., Deshusses, J. and Schaller, D.R. (1997). Polymerase chain reaction-based gene disruption in *Trypanosoma brucei*. *Molecular and Biochemical Parasitology* **87**, 113-115.

Gibson, W.C. (1989). Analysis of a genetic cross between *Trypanosoma brucei rhodesiense* and *T.b. brucei*. *Parasitology* **99**, 391-402.

Gibson, W.C. and Garside, L. (1991). Genetic exchange in *Trypanosoma brucei brucei*: variable chromosomal location of housekeeping genes in different trypanosome stocks. *Molecular and Biochemical Parasitology* **45**, 77-90.

Gibson, W.C., Garside, L. and Bailey, M. (1992). Trisomy and chromosome size changes in hybrid trypanosomes from a genetic cross between *Trypanosoma brucei rhodesiense* and *T. b. brucei*. *Molecular and Biochemical Parasitology* **52**, 189-200.

Gibson, W.C. and Whittington, H. (1993). Genetic exchange in *Trypanosoma brucei*: Selection of hybrid trypanosomes by introduction of genes conferring drug resistance. *Molecular and Biochemical Parasitology* **60**, 19-26.

Gibson, W.C. and Bailey, M. (1994). Genetic exchange in *Trypanosoma brucei*: evidence for meiosis from analysis of a cross between drug-resistant transformants. *Molecular and Biochemical Parasitology* **64**, 241-252.

Gibson, W.C. (1995). The significance of genetic exchange in trypanosomes. *Parasitology Today* **11**, 465-468.

Gibson, W.C., Winters, K., Mizen, G., Kearns, J. and Bailey, M. (1997). Intraclonal mating in Trypanosoma brucei is associated with outcrossing. *Microbiology* **143**, 909-920.

Gibson, W.C. and Stevens, J. (1999). Genetic exchange in the Trypanosomatidae. *Advances in Parasitology* **43**, 1-45.

Gibson, W.C. (2001). Molecular characterisation of field isolates of human pathogenic trypanosomes. *Tropical Medicine and International Health* **6**, 401-406.

Gregus, Z. and Gyurasics, A. (2000). Role of glutathione in the biliary excretion of the arsenical drugs Trimelarsen and Melarsoprol. *Biochemical Pharmacology* **59**, 1375-1385.

Gutteridge, W.E. (1985). Existing chemotherapy and its limitations. *British Medical Bulletin* **41**, 162-168.

Hall, N., Berriman, M., Lennard, N.J., Harris, B.R., Hertz-Fowler, C., Bart-Delabesse, E.N., Gerrard, C.S., Atkin, R.J., Barron, A.J., Bowman, S., Bray-Allen, S.P., Bringaud, F., Clark, L.N., Corton, C.H., Cronin, A., Davies, R., Doggett, J., Fraser, A., Grüter, E., Hall, S., Harper, A.D., . Kay, M.P., Leech, V., Mayes, R., Price, C., Quail, M.A., Rabbinowitsch, E., Reitter, C., Rutherford, K., Sasse, J., Sharp, S., Shownkeen, R., MacLeod, A., Taylor, S., Tweedie, A., Turner, C.M.R., Tait, A., Gull, K., Barrell, B. and Melville, S.E. (2003). The DNA sequence of chromosome 1 of an African trypanosome: gene content, chromosome organisation, recombination and polymorphism. *Nucleic Acids Research* **31**, 4864-4873.

Hawking, F. (1978). Suramin with special reference to onchocerchiasis. *Advances in Pharmacology and Chemotherpy* **15**, 289-322.

Hendriks, E.F.J., van Deursen, J., Wilson, M., Sarkar, M., Timms, M. and Matthews, K.R. (2000). Life-cycle differentiation in *Trypanosoma brucei*: molecules and mutants. *Biochemical Society Transactions* **28**, 531-536.

Hide, G., Cattand, P., LeRay, D., Barry, J. D. and Tait. A. (1990). The identification of *Trypanosoma brucei* subspecies using repetitive DNA sequences. *Molecular and Biochemical Parasitology* **39**, 213-226.

Hirumi, H., Hirumi, K., Moloo, S.K. and Shaw, M.K. (1992). *Trypanosoma brucei brucei*: In vitro production of metacyclic forms. *Journal of Protozoology* **39**, 619-627.

Hope, M., MacLeod, A., Melville, S., Tait, A. and Turner, C.M.R. (1999). Maintenance of diploidy (in megabase chromosomes) in *Trypanosoma brucei* after genetic exchange. *Molecular and Biochemical Parasitology* **104**, 1-9.

Hunt, M., Brun, R. and Kohler, P. (1994). Studies on compounds promoting the *in vitro* transformation of *Trypanosoma brucei* from bloodstream to procyclic forms. *Parasitology Research* **80**, 600-606.

Iraqi, F., Clapcott, S.J., Kumari, P., Haley, C.S., Kemp, S.J. and Teale, A.J. (2000). Fine mapping of trypanosomiasis resistance loci in murine advanced intercross lines. *Mammalian genome* **11**, 645-648.

Jenni, L., Marti, S., Schweizer, J., Betschart, B., Le Page, R.W.F., Wells, J.M., Tait, A., Paindavoin, P., Pays, E. and Steinert, M. (1986). Hybrid formation between African trypanosomes during cyclical transmission. *Nature* **322**, 173-175.

Kaminsky, R., Beaudoin, E. and Cunningham, I. (1987). Studies on the development of metacyclic *Trypanosoma brucei* spp. cultivated at 27°C with insect cell lines. *Journal of Protozoology* **34**, 372-377.

Kaminsky, R., Chuma, F. and Zweygarth, E. (1989). *Trypanosoma brucei brucei*: Expression of drug resistance *in vitro*. *Experimental Parasitology* **69**, 281-289.

Keiser, J. and Burri, C. (2000). Physico-chemical properties of the trypanocidal drug melarsoprol. *Acta Tropica* **74**, 101-104.

Krawchuk, M.D. and Wahls, W.P. (1999). High-efficiency gene targeting in *Schizosaccharomyces pombe* using a modular, PCR-based approach with long tracts of flanking homology. *Yeast* **15**, 1419-1427.

Lander, E. and Kruglyak, L. (1995). Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. *Nature Genetics* **11**, 241-247.

Lee, M.G.-S., Bihain, B.E., Russell, D.G., Deckelbaum, R.J. and Van der Ploeg, L.H.T. (1990). Characterisation of a cDNA encoding a cysteine-rich cell surface protein located in the flagellar pocket of the protozoan *Trypanosoma brucei*. *Molecular and Cellular Biology* **10**, 4506-4517.

Lee, M.G.-S., Russell, D.G., D'Alesandro, P.A. and Van der Ploeg, L.H.T. (1994). Identification of membrane-associated proteins in *Trypanosoma brucei* encoding an internal, EARLRAEE amino acid repeat. *Journal of Biological Chemistry* **269**, 8408-8415.

Lumsden, W.H.R., Cunningham, M.P., Webber, W.A.F., van Hoeve, K. and Walker, P.T. (1963). A method for the measurement of the infectivity of trypanosome suspensions. *Experimental Parasitology* **14**, 269-279.

MacLeod, A. (1999). PhD Thesis. Genetic analysis of *Trypanosoma brucei*.

Macleod, A., Turner, C.M.R. and Tait, A. (1999). A high level of mixed *Trypanosoma brucei* infections in tsetse flies detected by three hypervariable minisatellites. *Molecular and Biochemical Parasitology* **102**, 237-248.

MacLeod, A., Tweedie, A., Welburn, S.C., Maudlin, I., Turner, C.M.R. and Tait, A. (2000). Minisatellite marker analysis of Trypanosoma brucei: Reconciliation of clonal, panmictic, and epidemic population genetic structures. *Proceedings of the National Academy of Sciences, USA* **97**, 13442-13447.

MacLeod, A., Tait, A. and Turner, C.M.R. (2001). The population genetics of *Trypanosoma brucei* and the origin of human infectivity. *Philosophical Transactions of the Royal Society London Biological Science* **356**, 1035-1044.

Mäser, P., Sütterlin, C., Kralli, A. and Kaminsky, R. (1999). A nucleoside transporter from *Trypanosoma brucei* involved in drug resistance. *Science* **285**, 242-244.

Masiga, D. and Barrett, M.P. (2000). Sleeping Sickness. *African Health* (March) 5-9.

Masiga, D.K., Tait, A. and Turner, C.M. (2000). Amplified restriction fragment length polymorphism in parasite genetics *Parasitol Today* **16**, 350-353.

Matovu, E., Seebeck, T., Enyaru, J.C.K. and Kaminsky, R. (2001). Drug resistance in *Trypanosoma brucei* spp., the causative agents of sleeping sickness in man and nagana in cattle. *Microbes and Infection* **3**, 763-770.

Matovu, E., Stewart, M.L., Geiser, F., Brun, R., Mäser, P., Wallace, L.J., Burchmore, R.J., Enyaru, J.C.K., Barrett, M.P., Kaminsky, R., Seebeck, T. and de Koning, H.P. (2003). Mechanisims of arsenical and diamidines uptake and resistance in *Trypanosoma brucei. Eukaryotic Cell* **2**, 1003-1008.

Matthews, K.R. and Gull, K. (1994). Evidence for an interplay between cell cycle progression and the initiation of differentiation between life cycle forms of African trypanosomes. *Journal of Cell Biology* **125**, 1147-1156.

Matthews, K.R. and Gull, K. (1998). Identification of stage-regulated and differentiation-enriched transcripts during transformation of the African trypanosome from its bloodstream to procyclic form. *Molecular and Biochemical Parasitology* **95**, 81-95.

Matthews, K.R. (1999). Developments in the differentiation of *Trypanosoma brucei. Parasitology Today* **15**, 76-80.

Maynard-Smith, J., Smith, N.H., O'Rourke, M. and Spratt, B.G. (1993). How clonal are bacteria? *Proceedings of the National Academy of Sciences, USA* **90**, 4384-4388.

Metzenberg, S. and Agabian, N. (1994). Mitochondrial minicircle DNA supports plasmid replication and maintenance in nuclei of *Trypanosoma brucei*. *Proceedings of the National Academy of Sciences, USA* **91**, 5962-5966.

Nok, A.J. (2003). Arsenicals (melarsoprol), pentamidine and suramin in the treatment of human African trypanosomiasis. *Parasitology Research* **90**, 71-79.

Pal, A., Hall, B.S. and Field, M. (2002). Evidence for a non-LDL-mediated entry route for the trypanocidal drug suramin in *Trypanosoma brucei*. *Molecular and Biochemical Parasitology* **122**, 217-221.

Pépin, J. and Milford, F. (1994). The treatment of human African trypanosomiasis. *Advances in Parasitology* **33**, 1-47.

Philips, M.A. and Wang, C.C. (1987). A *Trypanosoma brucei* mutant resistant to α-difluoromethylornithine. *Molecular and Biochemical Parasitology* **22**, 9-17.

Priest, J.W. and Hajduk, S.L. (1994). Developmental regulation of mitocondrial biogenesis in *Trypanosoma brucei*. *Journal of Bioenergetics and Biomembranes* **26**, 179-191.

Raper, J., Portela, M.P.M., Lugli, E., Frevert, U. and Tomlinson, S. (2001). Trypanosome lytic factors: novel mediators of human innate immunity. *Current Opinion in Microbiology* **4**, 402-408.

Raz, B., Iten, Y., Grether-Buhler, R., Kaminsky, R. and Brun, R. (1997). The Alamar Blue assay to determine drug sensitivity of African trypanosomes (*T.b. rhodesiense* and *T.b. gambiense*) *in vitro*. *Acta Tropica* **68**, 139-147.

Ross, C.A. and Barns, A.M. (1996). Alteration to one of three adenosine transporters is associated with resistance to Cymelarsan in *Trypanosoma evansi*. *Parasitology Research* **82,** 183-188.

Sasse, J. (1998). PhD Thesis. University of Cambridge. The development of genetic markers for the *Trypanosoma brucei* genome.

Schweizer, J., Pospichal, H., Hide, G., Buchannan, N., Tait, A. and Jenni, L. (1994). Analysis of a new genetic cross between two East African *Trypanosoma brucei* clones. *Parasitology* **109,** 83-93.

Scott, A.G., Tait, A. and Turner, C.M.R. (1996). Characterisation of cloned lines of *Trypanosoma brucei* expressing stable resistance to MelCy and Suramin. *Acta Tropica* **60**, 251-262.

Scott, A.G., Tait, A. and Turner, C.M.R. (1997). *Trypanosoma brucei*: Lack of cross-resistance to Melarsoprol *in vitro* by Cymelarsan-resistant parasites. *Experimental Parasitology* **86**, 181-190.

Shen, S., Arhin, G.K., Ullu, E. and Tschudi, C. (2001). *In vivo* epitope tagging of *Trypanosoma brucei* genes using a one step PCR-based strategy. *Molecular and Biochemical Parasitology* **113**, 171-173.

Shirley, M.W. and Harvey, D.A. (2000). A genetic linkage map of the apicomplexan protozoan parasite *Eimeria tenella*. *Genome Research* **10**, 1587-1593.

Sibley, L.D., Mordue, D.G., Su, C., Robben, P.M. and Howe, D.K. (2002). Genetic approaches to studying virulence and pathogenesis in *Toxoplasma gondii*. *Philosophical Transactions of the Royal Society London Biological Science* **357**, 81-88.

Sidhu, A.B.S., Verdier-Pinard, D. and Fidock, D.A. (2002). Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by *pfcrt* mutations. *Science* **298**, 210-213.

Smith, D. F.and Parsons, M. (1996). Molecular Biology of Parasitic Protozoa. *Oxford University Press*, 6-26.

Sommer, J.M., Hua, S., Li, F., Gottesdiener, K.M. and Wand, C.C. (1996). Cloning by functional complementation in *Trypanosoma brucei*. *Molecular and Biochemical Parasitology* **76**, 83-89.

Sternberg, J., Tait, A., Haley, S., Wells, J.M., Le Page, R.W.F. Schweizer, J. and Jenni, L. (1988). Gene exchange in African trypanosomes: characterisation of a new hybrid genotype. Molecular and Biochemical Parasitology **27**, 191-200.

Sternberg, J., Turner, C.M.R., Wells, J.M., Ranford-Cartwright, L.C., Le Page, R.W.F. and Tait, A. (1989). Gene exchange in African trypanosomes: frequency and allelic segregation. *Molecular and Biochemical Parasitology* **34**, 269-280.

Sternberg, J. and Tait, A. (1990). Genetic exchange in African trypanosomes. *Trends in Genetics* **6**, 317-322.

Su, C., Howe, D.K., Dubey, J.P., Ajioka, J.W. and Sibley, L.D. (2002). Identification of quantitative trait loci controlling acute virulence in *Toxoplasma gondii*. *Proceedings of the National Academy of Sciences, USA* **99,** 10753-10758.

Su, X-z., Kirkman, L.A., Fujioka, H. and Wellems, T.E. (1997). Complex polymorphisms in a 330 kDa protein are linked to chloroquine-resistant *P. falciparum* in Southeast Asia and Africa. *Cell* **91,** 593-603.

Su, X-z., Ferdig, M.T., Huang, Y., Huynh, C.Q., Liu, A., You, J., Wootton, J.C. and Wellems, T.E. (1999). A genetic map and recombination parameters of the human malaria parasite *Plasmodium falciparum*. *Science* **286,** 1351-1353.

Swallow, B. (2000). Impacts of Trypanosomiasis on African Agriculture. Programme Against African Trypanosomiasis (PAAT). *PAAT Technical and Scientific Series,* **2.**

Tait, A. (1980). Evidence for diploidy and mating in trypanosomes. *Nature* **287,** 536-538.

Tait, A., Turner, C.M.R., Le Page, R.W.F. and Wells, J.M. (1989) Genetic evidence that metacyclic forms of *Trypanosoma brucei* are diploid. *Molecular and Biochemical Parasitology* **37,** 247-256.

Tait, A. and Turner, C.M.R. (1990). Genetic exchange in *T. brucei. Parasitology Today* **6,** 70-75.

Tait, A., Buchanan, N., Hide, G and Turner, C.M.R. (1993). Genetic recombination and karyotype inheritance in *Trypanosoma brucei* species. In: Genome *analysis of protozoan parasites*. Edited by Morgana, S.P., ILRAD, Nairobi, 93-107.

Tait, A., Buchanan, N., Hide, G. and Turner, C.M.R. (1996) Self-fertilisation in *Trypanosoma brucei*. *Molecular and Biochemical Parasitology* **76**, 31-42.

Tait, A.(2000). Tyrpanosomiases. *Molecular Epidemiology of Infectious Diseases*. Oxford University Press, 258-282.

Tait, A., Masiga, D., Ouma, J., MacLeod, A., Sasse, J., Melville, S., Lindegard, G., McIntosh, A. and Turner, C.M.R. (2002). Genetic analysis of phenotype in *Trypanosoma brucei*: a classical approach to potentially complex traits. *Philosophical Transactions of the Royal Society London Biological Science* **357**, (1417) 89-99.

Taylor, L.H., Katzer, F., Shiels, B.R. and Welburn, S.C. (2003). Genetic and phenotypic analysis of Tunisian *Theileria annulata* clones. *Parasitology* **126**, 241-252.

Tibayrenc, M., Kjellberg, F. and Ayala, F.J. (1990). A clonal theory of parasitic protozoa – the population structures of *Entamoeba, Giardia, Leishmania, Naegleria, Plasmodium, Trichomonas,* and *Trypanosoma* and their medical and taxonomical consequences. *Proceedings of the National Academy of Sciences, USA* **87**, 2414-2418.

Turner, C.M.R., Sternberg, J., Buchanan, N., Smith, E., Hide, G. and Tait, A. (1990). Evidence that the mechanism of gene exchange in *Trypanosoma brucei* involves meiosis and syngamy. *Parasitology* **101**, 377-386.

Turner, C.M.R., Aslam, N., Smith, E., Buchanan, N. and Tait, A. (1991). The effects of genetic exchange on variable antigen expression in *Trypanosoma brucei*. *Parasitology* **103**, 379-386.

Turner, C.M.R., Aslam, N. and Dye, C. (1995). Replication, differentiation, growth and the virulence of *Trypanosoma brucei* infections. *Parasitology* **111**, 289-300.

Turner, C.M.R. (1997). The rate of antigenic variation in fly-transmitted and syringe-passed infections of *Trypanosoma brucei*. *FEMS Microbiology Letters* **153**, 227-231.

Turner, C.M.R., McLellan, S., Lindergard, L.A.G., Bisoni, L., Tait, A. and MacLeod, A. (2004). Human infectivity trait in *Trypanosoma brucei*: Stability, heritability and relationship to SRA expression. *Parasitology* (In press).

Van den Abbeele, J., Claes, Y., Van Bockstaele, D., LeRay, D. and Coosemans, M. (1999). *Trypanosoma brucei* spp. Development in the tsetse fly: characterisation of the post-mesocyclic stages in the foregut and proboscis. *Parasitology* **118**, 469-478.

Vanhamme, L., Paturiaux-Hanocq, F., Poelvoorde, P ., Nolan, D.P., Lins, L., Van Den Abbeele, J., Pays, A., Tebabi, P., Van Xong, H., Jacquet, A., Moguilevsky, N., Dieu, M., Kane, J.P., De Baetselier, P., Brasseur, R. and Pays, E. (2003). Apolipoprotein L-I is the trypanosome lytic factor of human serum resistance. *Nature* **422**, 83-87.

Vickerman, K. (1985). Developmental cycles and biology of pathogenic trypanosomes. *British Medical Bulletin* **41**, 105-114.

Vickerman, K., Tetley, L., Hendry, K.A.K. and Turner, C.M.R. (1988). Biology of African trypanosomes in the tsetse fly. *Biology of the Cell* **64**, 109-119.

Wang, C.C. (1995). Molecular mechanisms and therapeutic approaches to the treatment of African trypanosomiasis. *Annual Review of Pharmacology and Toxicology* **35**, 93-127.

Wang, C.C. (1997). Validating targets for antiparasite chemotherapy. *Parasitology* **114** (supplement), 31-34.

Welburn, S.C. and Maudlin, I. (1997). Control of *Trypanosoma brucei* infections in tsetse, *Glossina morsitans*. *Medical Veterinary Entomology* **11**, 286-289.

Wellems, T.E., Walker-Jonah, A. and Panton, L.J. (1991). Genetic mapping of the chloroquine-resistance locus on *Plasmodium falciparum* chromosome 7. *Proceedings of the National Academy of Sciences, USA* **88**, 3382-3386.

Werbovetz, K., Bhattacharya, G., Salem, M., Barszcz, T. and Delfin, D. (2003). Selective antimitotic agents against kinetoplastid parasites. *American Society of Tropical Medicine and Hygiene Meeting*, Philadelphia, USA.

Williamson, J. (1970). Review of chemotherapeutic and chemoprophylactic agents. In *'The African Trypanosomiasis'* (Mulligan, H.W., Ed) Allen and Unwin, London, 125-224.

Yuan, Z., Davis, M.J., Zhang, F. and Teasdale, R.D. (2003). Computational differentiation of N-terminal signal peptides and transmembrane helices. *Biochemical and Biophysical Research Communications* **312**, 1278-1283.

Zhang, Z.Q., Giroud, C. and Baltz, T. (1992). *In vivo* and *in vitro* sensitivity of *Trypanosoma evansi* and *T. equiperdum* to diminazene, suramin, MelCy, quinapyramine and isometamidium. *Acta Tropica* **50**, 101-110.