Studies on a Thiol-Dependent Reductase and Ascorbate Metabolism of *Leishmania*

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Abstract

The intracellular protozoan parasite *Leishmania* causes leishmaniasis, a disease which is most prevalent in tropical and sub-tropical countries where it infects some two million people every year and kills around 60,000 of them. For decades pentavalent antimonial compounds have been the standard first-line drugs used to treat the disease and this remains the case despite increasing reports of drug-resistance. The mode of action of these drugs is not entirely understood, although it is generally accepted that *in vivo* reduction of the compounds from the pentavalent to a trivalent form is required for antileishmanial activity. The site of antimonial conversion and whether the reaction is catalysed by an enzyme remain controversial points. However, it was recently reported that *L. donovani* amastigotes were capable of reducing pentavalent antimonials to the trivalent form and that drug-resistant parasites were deficient in this activity, suggesting that a parasite enzyme did mediate drug toxicity. The identity of such an enzyme was investigated in this study.

Arsenical and antimonial compounds are similar and several classes of proteins that exhibit arsenate reductase activity have been previously identified in other organisms. Whether *Leishmania* possessed an enzyme akin to one of these was assessed by attempting to purify enzymes from parasite lysates and by searching the *L. major* genome database for similar sequences to the arsenate reductases. The latter approach was successful and a gene fragment was identified that shared similarity with omega glutathione S-transferases (oGSTs), a class of glutaredoxin-like GSTs which are capable of reducing pentavalent methylated arsenicals *in vitro*. The sequence of the complete *L. major* gene was elucidated by 5' RACE, and was found to encode a protein twice the expected size with similar 3' and 5' halves. The protein was named thiol-dependent reductase, or TDR1. Active recombinant protein was successfully produced and its biochemical activities were found to coincide with oGSTs: TDR1 was capable of reducing pentavalent arsenical and antimonial compounds to trivalent species, and possessed thioltransferase and dehydroascorbate reductase activities usually associated with glutaredoxins. TDR1, which was shown to probably reside in the parasite cytosol but may also be secreted, was found to be more abundant in amastigote than promastigote forms, which correlates with the antileishmanial stage-specificity of pentavalent antimonials. *L. major* TDR1 knockout mutants were generated, and the protein was also over-expressed in parasites. Both these genetic manipulations resulted in mutants with enhanced infectivity.

TDR1 knockout parasites were more susceptible than wild type parasites to paraquat, which induces the production of intracellular superoxide. As its glutaredoxin-like *in vitro*

activities suggest, this implies TDR1 has a role in protecting the parasites from oxidative stress, although re-expression of TDR1 did not reinstate resistance. Whether TDR1 has a role in susceptibility to pentavalent antimonials was investigated by studying the effect of the drug on *L. major* in macrophages. There appeared to be little difference in the effect of the drug on TDR1 knockout, over-expressing and wild type parasites, although variation in infectivity to macrophages and the insensitivity of *L. major* to the drug complicated the situation.

Whether trivalent antimonials could be oxidised to non-trivalent species by hydrogen peroxide was assessed. This was indeed found to be the case, demonstrating that in principle antimonial metabolism may be more complex than straightforward reduction from the pentavalent to trivalent form. However, the physiological relevance of this finding is uncertain due to oxidation of the antimonials being inhibited by glutathione.

The dehydroascorbate reductase activity of TDR1 was of interest as the presence of an enzyme capable of maintaining ascorbate in its reduced form may imply that this low-molecular weight thiol is important in the parasite. The recent identification of an ascorbate-dependant peroxidase in *Leishmania* added further credence to this hypothesis. Many organisms produce ascorbate *de novo* although, due to the loss of an important synthesis enzyme, humans cannot and have to scavenge ascorbate from their diet. If *Leishmania* does require ascorbate and rely on *de novo* production, enzymes that mediate synthesis could feasibly be exploitable drug targets. Whether *Leishmania* was capable of producing ascorbate and its importance in the parasite was investigated. The *L. major* genome database was searched for sequences similar to those of enzymes known to be involved in ascorbate synthesis in other organisms. Several candidate protein sequences were identified including that of one which is similar to L-gulono lactone oxidase (GLO), the enzyme that mediates the final step in ascorbate production in a variety of organisms and is the protein humans no longer possess. This *L. major* sequence was named LmGLO. *L. major* LmGLO knockout mutants were generated and the protein was also over-expressed in parasites. While over-expression resulted in parasites being more infective, loss of LmGLO resulted in decreased infectivity, both *in vitro* and *in vivo*. In addition, LmGLO knockout promastigotes displayed a slight growth defect. Although these results need extending, they suggest that *Leishmania* parasites do indeed synthesise ascorbate and that this ability is important for optimal virulence and infectivity to mammals.

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Denton, H., J. C. McGregor and G. H. Coombs (2004). "Reduction of anti-leishmanial pentavalent antimonial drugs by a parasite-specific thiol-dependent reductase, TDR1." *Biochem J* **381**(Pt 2): 405-12

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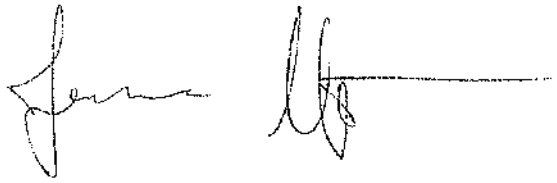
This thesis is dedicated to my granny, Catherine McGinley, who would have been the proudest of all.

Declaration

The research reported in this thesis is my own, original work, except where otherwise stated, and has not been submitted for any other degree.



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Abbreviations

Å	angstrom
A	adenosine
AAD	Ascorbate-dependent dioxygenase
ALO	D-arabinono-1,4-lactone oxidase
ALP	alkyl-lysophospholipid
APX	ascorbate-dependent peroxidase
ARA1	D-arabinose dehydrogenase
As(III)	trivalent arsenical
As(V)	pentavalent arsenical
ATP	adenosine triphosphate
bp	base pair
BPR	bromopyrogallol red
°C	degrees celcius
C	cytosine
cDNA	complimentary DNA
CDNB	1-chloro-2,4-dinitrobenzene
cm	centimetre
CS	cysteine synthase
DAPI	4',6-diamidino-2-phenylindole
DEPC	diethylpyrocarbonate
DHA	dehydroascorbate
DHAR	dehydroascorbate reductase
DHFR	dihydrofolate reductase
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E-64	(2*S*,3*S*)-3-(*N*-{(*S*)-1-[*N*-(4-guanidinobutyl)carbamoyl]3-methylbutyl}carbamoyl)oxirane-2-carboxylic acid
EDTA	ethylenediamine tetraacetic
ER	endoplasmic reticulum
g	gravity acceleration
g	gram
G	guanine
GALDH	L-galactono-1,4-lactone dehydrogenase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GLO	L-gulonolactone oxidase
GR	glutathione reductase
GRX	glutaredoxin
GSH	glutathione
GSSG	glutathione disulphide
GST	glutathione S-transferase
h	hours
H_2O_2	hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIFCS	heat-inactivated foetal calf serum
HRP	horseradish peroxidase
IC_{50}	50% maximal inhibitory concentration
IPTG	isopropylthiol-β-D galactoside
KAT	potassium antimony tartrate
kb	kilobase
kD	kiloDalton
kDNA	kinetoplastid DNA

KO	knockout
kV	kilovolt
LmGLO	*Leishmania major* L-gulonolactone oxidase
µCi	microcurie
µF	microfarad
ug	microgram
µl	microlitre
µm	micrometer
µM	micromolar
M	molar
MDHA	monodehydroascorbate
MDHAR	monodehydroascorbate reductase
mg	milligram
MGA	meglumine antimoniate
ml	millilitre
mm	millimetre
mM	millimolar
MMA(V)	methylarsonate
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger RNA
MTAP	methylthioadenosine phosphorylase
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
ng	nanogram
nm	nanometre
OD	optical density
oGST	omega GST
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
PNP	purine nucleoside phosphorylase
RACE	rapid amplification of cDNA ends
ROS	reactive oxygen species
RNA	ribonucleic acid
rTDR1	recombinant TDR1
Sb(III)	trivalent antimonial
Sb(V)	pentavalent antimonial
SD	standard deviation
SDS	dodium dodecylsulphate
SE	standard error
SSC	saline sodium citrate
SSG	sodium sticogluconate
T	thymine
TCEP	tris(2-carboxyethyl)phosphine hydrochloride
TDR1	thiol-dependent reductase 1
TK	transketolase
TR	trypanothione reductase
TRX	tryparedoxin
TS	trypanothione synthetase
$T(SH)_2$	trypanothione
TS_2	trypanothione disulphide
V	volt
WT	wild type
X-gal	5-bromo-4-chloro-3-indolyl- isopropylthiol-β-D galactoside

1 Introduction

1.1 The *Leishmania* parasite

The genus *Leishmania* belongs to the family *Trypanosomatidae*, of the order Kinetoplastida, a reference to the unusual kinetoplast organelle that the parasite contains in its single mitochondrion. Over 20 species of the protozoan parasites are known to exist, which cause different manifestations of the disease leishmaniasis in a variety of mammalian hosts, such as canids and rodents, as well as humans. *Leishmania* parasites are most closely related to trypanosomatids such as *Trypanosoma brucei* and *Trypanosoma cruzi,* the causative agents of African sleeping sickness and Chagas disease, respectively. The vector responsible for spreading the eukaryotic *Leishmania* parasite is the female sand fly of the genus *Phlebotomus* in the old world and *Lutzomyia* in the new world. *Leishmania* parasites cause the disease leishmaniasis, which is most prevalent in tropical and sub-tropical regions where the sand flies thrive, and in more than 80 countries the parasite is endemic.

1.1.1 The *Leishmania* life cycle

The parasite can exist in several different states during its life cycle, which is illustrated in figure 1.1. While in the sand fly vector they exist as promastigotes, transforming from procyclic to metacyclic forms. The motile promastigotes are an elongated oval shape with an anterior flagellum, approximately 10-20 μM in length, which replicate by asexual reproduction. The infectious metacyclic forms differ from procyclics in several ways: they have narrower bodies and longer flagella, are more motile, do not divide and have different biochemical compositions (Mallinson and Coombs 1989) and protein expression profiles (Nugent *et al.,* 2004). As such, they are considered to be a distinct life-cycle stage. Following metacyclogenesis, the promastigotes migrate from the midgut to the proboscis of the insect vector, and are transmitted to the animal host when the sand fly bites. Thereafter the promastigotes are phagocytosed by host macrophages (and other cells (Bogdan *et al.,* 2000)) where they transform to amastigote forms and proliferate, dividing again by binary fission, in the phagolysosome. Amastigotes are morphologically and biochemically distinct from promastigotes: they are much smaller (2-6 μm in diameter), immobile, ovoid forms lacking prominent flagella, and have very different protein expression profiles (Walker *et al.,* 2006). Parasite-containing macrophages rupture and release the amastigotes, which then go on to infect more cells. Amastigotes are then taken up in a subsequent sandfly bite. Once in the vector, the amastigotes transform to procyclic

promastigotes, recommencing the cycle. *Leishmania* promastigotes can be grown in culture, facilitating research into the parasites. In addition, amastigotes of some species can also be grown axenically (Gupta *et al.*, 2001).

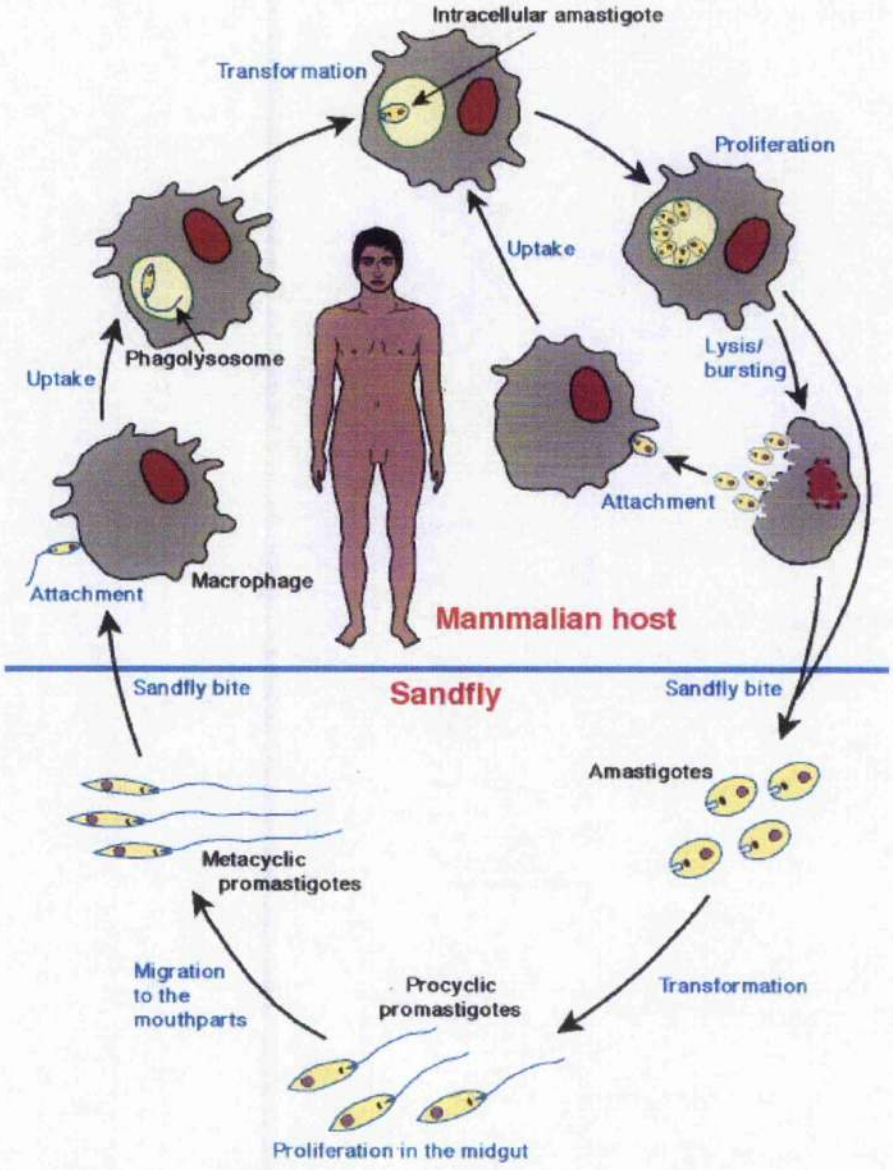


Figure 1.1: Life cycle of *Leishmania*. The amastigote (intracellular in mammalian host) and promastigote (extracellular in insect vector) life cycle stages are depicted. This image is taken from the website www.wehi.edu.au/media/images/leishmania_cycle.gif.

1.1.2 The *Leishmania* genome and regulation of gene expression

The genomes of several *Leishmania* species, which are diploid organisms, are currently being sequenced and the complete, annotated genome sequence of *Leishmania major* Freidlin was recently published (Ivens *et al.*, 2005). *L. major* has a 32.8 megabase haploid genome divided into 36 chromosomes and is predicted to contain 8272 protein-encoding genes and 911 RNA genes. In addition, the kinetoplast contains its own DNA (kDNA) which is the equivalent of mitochondrial DNA. However, kDNA is arranged in an unusual structure comprising catenated minicircles of which there are several thousand per kinetoplast, and maxicircles of which there are several dozen. Approximately 20 proteins are encoded by maxicircle kDNA, most of which are thought to be mitochondrial proteins involved in energy transduction.

Leishmania have an unusual method of gene expression with chromosomal protein-encoding genes being arranged in directional gene clusters (Myler *et al.*, 1999) which undergo polycistronic transcription (Worthey *et al.*, 2003). Accordingly, mRNA abundance does not necessarily reflect the level of a given protein in the parasites (Holzer *et al.*, 2006; McNicoll *et al.*, 2006), meaning that analysis of the expression level of *Leishmania* proteins by northern blotting is problematic. Like in other trypanosomatids, a conserved RNA sequence of 39 nucleotides, which is known as the splice-leader sequence, is trans-spliced onto the 5' end of all *Leishmania* mRNAs; the 3' end of most mRNAs are polyadenylated by trans-splicing also. These events are required for successful translation. Gene expression and resulting protein levels in trypanosomatids are thought to be mediated in several non-transcriptional ways: RNA degradation, control of translation and post-translational events are all thought to contribute, as reviewed in Clayton, 2002.

1.1.3 Redox regulation in trypanosomatids

Upon infection of an organism, parasitic protozoa encounter high levels of reactive oxygen species due to the oxidative burst response of the host's immune system and it is therefore of interest that trypanosomatids exhibit atypical mechanisms for dealing with oxidative stress. Distinctive thiol-based systems for regulating the redox environment have evolved in many protozoan parasites (reviewed in Muller *et al.*, 2003b). In trypanosomatids these differences are underpinned by the presence of two unusual thiols that have been identified: ovothiol and trypanothione ($T(SH)_2$). The precise function of ovothiol is not yet fully understood as, despite its abundance in *Leishmania*, it is not known to participate in any enzymatic reactions and may simply act as a scavenger of reactive oxygen species

(ROS) (Ariyanayagam and Fairlamb 2001). Meanwhile, $T(SH)_2$ has a pivotal role in the thiol-based redox metabolism of *Leishmania* because it is responsible for keeping other thiols reduced. Although trypanosomatids also contain high levels of the almost ubiquitous thiol glutathione (GSH), they lack glutathione reductase which in other systems reduces glutathione disulphide (GSSG), the oxidised form of GSH. Instead they possess trypanothione reductase (TR), an essential enzyme in *Leishmania* (Tovar *et al.*, 1998), which regenerates oxidised trypanothione (TS_2) to the reduced $T(SH)_2$, which in turn reduces GSSG (Fairlamb *et al.*,, 1985). Although this reaction occurs non-enzymatically, the *Trypanosoma cruzi* enzyme Tc52 is also able to catalyse the reduction of GSSG by $T(SH)_2$ (Moutiez *et al.*,, 1995). Ovothiol is also dependant on $T(SH)_2$ for maintenance in its reduced state (Ariyanayagam and Fairlamb 2001).

$T(SH)_2$ is comprised of two molecules of GSH that are linked by a molecule of spermidine, the conjugation of which is catalysed by trypanothione synthetase in *L. major* (Oza *et al.*, 2005), *T. brucei* (Oza *et al.*, 2003) and *T. cruzi* (Oza *et al.*, 2002). In the non-pathogenic insect trypanosomatid *Crithidia fasiculata*, which has been used as a model organism for investigating $T(SH)_2$ synthesis, two enzymes were thought to regulate the formation of $T(SH)_2$: glutathionylspermidine synthetase and trypanothione synthetase (TS) (Tetaud *et al.*, 1998). However, more recent findings have shown that only the latter enzyme is required for $T(SH)_2$ synthesis (Comini *et al.*, 2005) as is the case in pathogenic trypanosomatids. $T(SH)_2$ participates in many enzymatic and non-enzymatic reactions including the reduction of dehydroascorbate (Krauth-Siegel and Ludemann 1996) and acts as a co-factor with trypanothione S-transferases (which have been postulated to replace glutathione S-transferases in trypanosomatids) in the detoxification of xenobiotics (Vickers *et al.*, 2004). In addition $T(SH)_2$ reduces tryparedoxin, a trypanosomatid-specific thioredoxin-like protein which reduces peroxiredoxins which in turn enzymatically detoxify hydroperoxides (Nogoceke *et al.*, 1997). Peroxidases are thought to be of particular importance in some parasitic protozoa due to the absence of catalase (Muller *et al.*, 2003b). The reducing equivalents for DNA synthesis are also provided by $T(SH)_2$ as it reduces ribonucleotide reductase (the enzyme required to synthesise nucleotide precursors), either directly or via tryparedoxin (Dormeyer *et al.*, 2001).

Reflecting the diversity of the reactions $T(SH)_2$ is involved in and its role in regulating the redox environment of the parasite, enzymes known to be involved in $T(SH)_2$ synthesis and regeneration – namely TR and TS – are thought to be essential in trypanosomes. When RNAi was performed on *T. brucei* parasites resulting in reduced levels of TS, growth defects and increased sensitivity to oxidative stress, together with elevated levels of TR,

were observed (Comini *et al.*, 2004; Ariyanayagam *et al.*, 2005). Attempts to create *L. donovani* TR null-mutants have been unsuccessful and both *L. donovani* and *T. brucei* parasites engineered to have reduced levels of the protein exhibited diminished viability *in vivo* (reviewed in Krauth-Siegel and Inhoff, 2003). These findings, coupled with the parasite-specific nature of the thiol, has lead to $T(SH)_2$ metabolism being considered a valid drug-target and inhibitors of both TR and TS being sought. Indeed trivalent antimonial drugs, the reduced form of the most common first-line treatment against leishmaniasis, have been shown to inhibit TR *in vitro* (Cunningham and Fairlamb, 1995) and both the administered pentavalent antimonials and the trivalent form exert effects suggestive of inhibition of TR *in vivo* (Wyllie *et al.*, 2004).

1.2 Leishmaniasis

The World Health Organisation collates epidemiological information on leishmaniasis and much of the forthcoming information was obtained from their relevant website at http://www.who.int/leishmaniasis/. Disease resulting from infection with the parasite *Leishmania* has three predominant forms: visceral, cutaneous and mucocutaneous leishmaniasis. Visceral leishmaniasis is the most severe form: symptoms include fever, diarrhoea, hepatosplenomegaly, pancytopenia, epistaxis, cachexia and peripheral lymphodenopathy, and, if left untreated, this form of the infection is almost always fatal. Visceral leishmaniasis affects many different bodily organs; macrophages become infected with parasites throughout the reticuloendothelial system and they ultimately reach the liver, spleen and bone marrow. The incubation period of the disease can be months or even longer, with death usually occurring in untreated sufferers approximately two years later. Infection with several species of *Leishmania* causes the visceral form of the disease; these include the old world species *L. tropica*, *L. donovani*, and *L. infantum*, and *L. chagasi* in the new world (which is very similar to *L. infantum*).

The least severe form of the disease is cutaneous leishmaniasis, a form that affects the skin. It causes isolated ulcers on exposed parts of the body, which are often disfiguring and leave scars. However, given enough time, spontaneous healing can occur which can result in leishmaniasis immunity in the patient. Cutaneous leishmaniasis is caused by infection with old world species *L. major*, *L. tropica* and *L. aethiopica*; and new world species *L. mexicana*, *L. amazonensis*, *L. braziliensis*, *L. peruviana*, *L. guyanensis* and *L. panamensis*. *L. aethiopica* and *L. amazonensis* can also cause a more severe form of the skin disease known as diffuse cutaneous leishmaniasis which requires treatment. As well as a lesion

forming at the site of infection, chronic satellite lesions occur as the parasites metastasise to other areas of the skin.

The third distinct form of the disease, known as mucocutaneous leishmaniasis, also affects the skin but also facial mucosal tissue. A cutaneous infection can descend into the mucocutaneous variety months after the initial lesion has healed. Infection can lead to complete degradation of the nose, mouth and throat, which is both highly debilitating and disfiguring, and fatality can result from secondary infections. Mucocutaneous leishmaniasis is caused by infection with *L. braziliensis* and, less frequently, *L. panamensis*; accordingly this form of the disease is only prevalent in the new world. Although each form of the disease is distinct, it should be noted that a cutaneous or mucosal infection can occasionally deteriorate into visceral leishmaniasis, and that sufferers of *L. donovani*-induced visceral leishmaniasis can develop cutaneous lesions.

Leishmaniasis is prevalent in 88 countries worldwide and of these, declaration is obligatory in just 32. Infections are common in remote areas where access to medical care and facilities is limited. Consequently, incidence of the disease is under-reported and therefore it is difficult to determine the global burden and what the mortality rate is. It is currently estimated that there are approximately 50,0000 new cases of visceral leishmaniasis and 2 million new cases of cutaneous leishmaniasis each year; although in 2001 only 600,000 cases were reported in total. Reportedly, 59,000 people died from visceral leishmaniasis in 2001. It was estimated that 2.4 million DALYs (disability-adjusted life years, the number of years lost to disability and premature mortality) were lost due to leishmaniasis (http://www.who.int/tdr/diseases/leish/). However, these numbers are subject to fluctuation due to epidemics: during an outbreak in Sudan in the early 1990s, Médecins sans Frontières reported that 100000 people died from leishmaniasis, which was more than 10% of the at-risk population.

As mentioned, leishmaniasis occurs where the sand fly vectors thrive: mainly in tropical and sub-tropical regions of Europe, Africa, Asia and Central and South America (figure 1.2). Among these are 16 European countries; the remainder are developing nations, reflecting the nature of leishmaniasis as being a disease of the poverty-stricken. Over 90% of visceral infections reportedly occur in Bangladesh, Brazil, India, Nepal and Sudan and over 90% of reports of cutaneous disease are in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria. Unfortunately, the most recent comprehensive statistics on leishmaniasis epidemiology – summarised in the 2002 World Health Report – are from 2001, and therefore changes in the distribution and incidence of leishmaniasis in recent years are

unknown. However, it is accepted that leishmaniasis is an increasing problem in many parts of the world (Desjeux, 2001). This is illustrated by the increase in cutaneous leishmaniasis cases in certain countries between the 1990s and the 2000s: in 1998 there were 21800 cases reported in Brazil but in 2002 the figure was 40000, while reported incidents of the disease in Kabul, Afghanistan rose from 14200 in 1992 to 65000 in 2002. The same is true of visceral leishmaniasis: the number of cases in north-eastern Brazil rose from 1840 in 1998 to 6000 in 2002. In recent years there has been an increase in the number of infections in Southern Europe, as is discussed below.

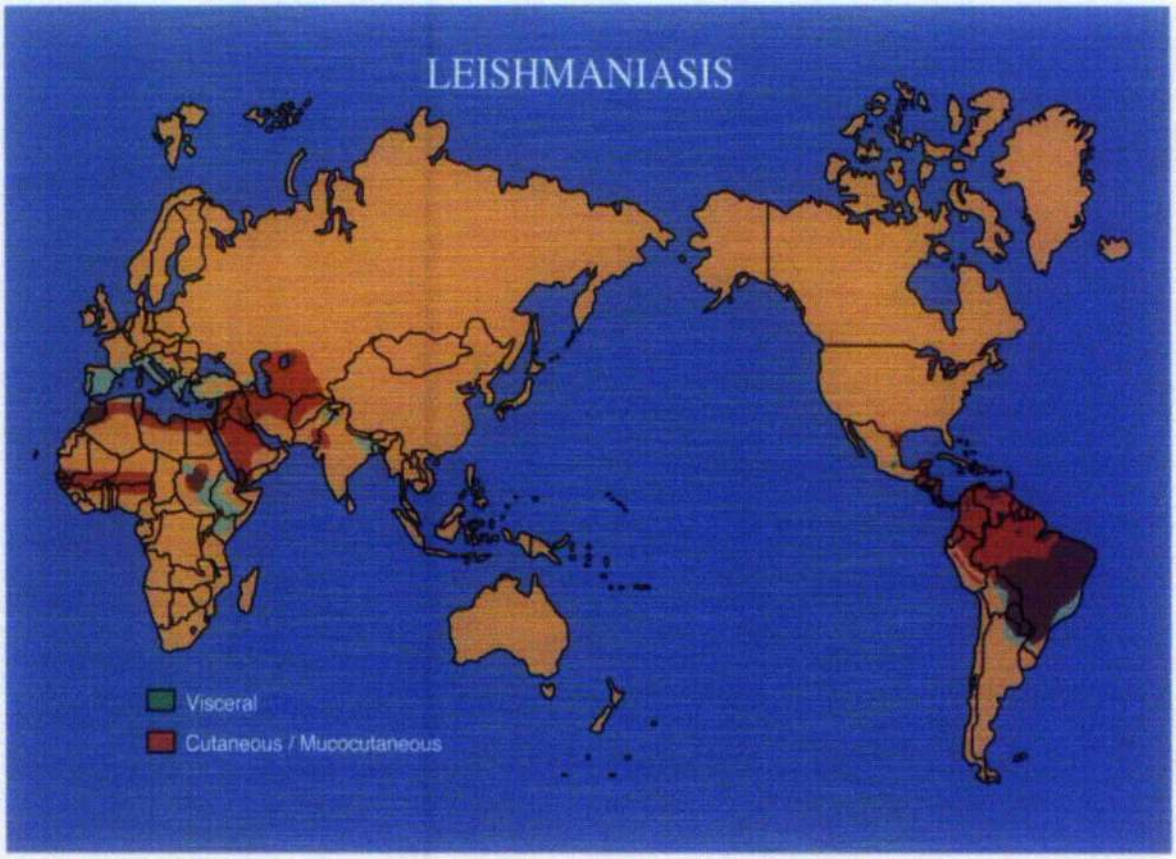


Figure 1.2: Global distribution of leishmaniasis. Regions where the visceral form of the disease persists is in green and areas where the cutaneous and mucocutaneous forms exist are in red. The image is taken from the website http://www.wehi.edu.au/media/images/handman/world_map.jpg.

There are several reasons for the changing epidemiology of the disease (Desjeux, 2004). Immuno-compromised patients are at greater risk of developing clinical leishmaniasis (as opposed to passive infections that go undetected) than healthy individuals and this has resulted in HIV co-infection (Desjeux *et al.*, 2001). This has been a particular problem with increasing infections of recurring visceral leishmaniasis in parts of Southern Europe

including Spain, Italy, France and Portugal. However, improvements in HIV chemotherapy have led to a concomitant decrease in leishmaniasis in HIV sufferers in Europe (Lopez-Velez, 2003). In many developing countries HIV/leishmaniasis co-infection remains a growing problem. Another reason for the change in disease pattern is population migration: urbanisation and military unrest have both caused recent large-scale movement of people from uninfected areas, who lack innate immunity, to endemic regions. The challenges presented by the increasing prevalence and changing distribution of the disease mean that effective chemotherapy against leishmaniasis is imperative.

1.3 Chemotherapy and resistance

There are several *Leishmania* vaccine candidates currently under investigation that include the use of whole killed cells, live attenuated cells, recombinant *Leishmania* proteins and peptides and DNA vaccines (reviewed in Ghosh and Bandyopadhyay, 2003). However, no prophylactic is currently available and accordingly, effective chemotherapy is of utmost importance. Treatment of leishmaniasis is dependant upon several factors including the economic situation in the country where the infection has occurred, whether drug resistance is a problem in the area, and what form of leishmaniasis is present in the individual. Over 90% of cutaneous leishmaniasis infections heal over time and accordingly are often not treated (Davies *et al.*, 2003) while visceral leishmaniasis is always treated if possible, due to infection being fatal. Mucocutaneous infections are usually treated as this form can be damaging also. There are several different chemotherapeutic options available to treat leishmaniasis, as well as promising new drug candidates. These are summarised in table 1.1, and discussed in this section. Widespread drug resistance has been reported in parts of Northern India to pentavalent antimonials and this has presented a further challenge to leishmaniasis treatment; accordingly, issues surrounding drug resistance are addressed in this section. Appreciating why the drugs are toxic to the parasites is crucial in understanding how they develop ways to evade the toxicity and this is also discussed. Several promising new compounds are currently in the advanced stages of clinical trials and, as the potential future of leishmaniasis chemotherapy, these too are described.

Type of leishmaniasis	Status of drug	Drugs available
visceral	first-line	pentavalent antimonials (sodium stibogluconate (Pentostam), meglumine antimoniate (Glucantime)); amphotericin B; pentamidine; miltefosine (India only)
	clinical trials	miltefosine; paromomycin; sitamaquine
cutaneous	first-line	pentavalent antimonials (sodium stibogluconate (Pentostam), meglumine antimoniate (Glucantime)); amphotericin B; pentamidine; miltefosine (Columbia only)
	clinical trials	miltefosine; paromomycin; azoles

Table 1.1: Drugs for leishmaniasis treatment. Table redrawn, with modifications, from a previous report (Croft *et al.*, 2006).

1.3.1 Pentavalent antimonials

There are two pentavalent antimonial compounds that have been used to treat leishmaniasis for over 60 years: sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime), as well as generic varieties made in India and China. Antimonial compounds remain the standard first-line treatment for both visceral and cutaneous forms of leishmaniasis in almost all parts of the world, although emerging drug-resistance has resulted in the licensing of Miltefosine in some areas (see section 1.3.2). Pentavalent antimonials will be discussed in detail in section 1.4.

1.3.2 Miltefosine

Miltefosine (hexadecylphosphocholine) is a relatively new antileishmanial drug that has the major advantage of being administered orally. A member of a family of compounds called alkyl-lysophospholipids (ALPs), it was originally developed as an anti-cancer drug and was found to have an anti-proliferative effect on *Leishmania in vitro* and *in vivo* (Croft *et al.*, 1987). It is active against many *Leishmania* species although there is variation in the sensitivity of these: *L. donovani* has been shown to be the most susceptible (Escobar *et al.*, 2002; Yardley *et al.*, 2005). Currently in phase IV clinical trials, Miltefosine has been used successfully to treat visceral leishmaniasis with phase II clinical trial cure rates of 95% and 98% (Jha *et al.*, 1999; Sundar *et al.*, 1999) and has been licensed for use in India since 2002. Following reports showing that Miltefosine was toxic against cutaneous leishmaniasis likely to be caused by *L. panamensis* (Soto *et al.*, 2001), the drug has been

recently licensed for use against this form of the disease in Columbia. A topical formulation of Miltefosine (Miltex) is also effective in treating cutaneous leishmaniasis (Schmidt-Ott *et al.,* 1999). Miltefosine may be useful in treating infections of other parasites including *Trypanosoma cruzi* (Croft *et al.,* 1996; Santa-Rita *et al.,* 2000), *Entamoeba histolytica* (Seifert *et al.,* 2001) and *Acanthamoeba* spp. (Walochnik *et al.,* 2002).

The mode of action of Miltefosine has not been fully elucidated. Most work in this area has been carried out on cancerous mammalian cells as opposed to trypanosomatids and it is not known if the mechanism of toxicity in these is similar (Croft *et al.,* 2003). It is known that Miltefosine induces apoptosis in cells (Konstantinov *et al.,* 1998) and more recently this has been shown to occur in both *L. donovani* promastigotes (Paris *et al.,* 2004) and amastigotes (Verma and Dey, 2004). The induction of apoptosis in mammalian cells has been attributed to several mechanisms. These include inhibition of phosphocholine biosynthesis by disrupting the translocation of CTP:phosphocholine-cytidylyltransferase (Geilen *et al.,* 1992); stimulation of the stress-activated protein kinase/c-Jun NH_2-terminal kinase (SAPK/JNK) pathway (Ruiter *et al.,* 1999); disruption of signal transduction via inhibition of protein kinase C (Uberall *et al.,* 1991) and stimulation of cellular ceramide formation (Wieder *et al.,* 1998). However the role of inhibition of phosphatidylcholine synthesis in apoptosis has been recently questioned due to apoptosis being induced by Miltefosine in cells impaired in phosphatidylcholine synthesis by an alternative mechanism (van der Sanden *et al.,* 2004). Significantly less research has been carried out into the affect of ALPs on trypanosomatids; the limited investigations that have been conducted were reviewed recently by Croft *et al.,* 2003. The treatment of *Trypanosoma cruzi* with various ALPs including Miltefosine caused extensive blebbing of the flagellar membrane (Santa-Rita *et al.,* 2000) and the affect of Miltefosine on parasite membrane lipids has been a focus of interest. It was originally shown that Miltefosine affected ether-lipid metabolism and glycosylphosphatidylinositol (GPI) anchor biosynthesis (Lux *et al.,* 1996). The same group have since shown that the drug inhibits alkyl-specific-acyl-CoA acyltransferase, an enzyme involved in lipid-remodelling (Lux *et al.,* 2000). Recently it has been reported that laboratory-derived Miltefosine-resistant *L. donovani* promastigotes displayed altered membrane lipid composition (Rakotomanga *et al.,* 2005).

Resistance to ALPs has so far only been observed in the laboratory, although this is expected to occur in the field in time due to several factors (Berman *et al.,* 2006). The drug has a narrow therapeutic index and long half-life (Sundar, 2001b): both are factors considered to favour the emergence of resistance. In recent domiciliary clinical trials the

relapse rate doubled as compared to supervised in-patient trials (Sundar and Murray, 2005), suggesting that non-supervised Miltefosine administration could result in conditions that would favour resistance. Moreover, the ease of generating resistance in the laboratory has given cause for concern (Seifert *et al.,* 2003). It has been suggested that in order to prevent Miltefosine resistance becoming problematic in the field, the drug should always be used in combination with a second unrelated antileishmanial compound such as paromomycin or amphotericin B (Bryceson 2001).

Several different mechanisms of Miltefosine resistance have been proposed in *Leishmania,* as reviewed in Croft *et al.,* 2006. *Leishmania tropica* engineered to over-express a P-glycoprotein-like transporter displayed more than nine times increased tolerance to Miltefosine than wild type parasites (Perez-Victoria *et al.,* 2001). The engineered parasites showed a reduced accumulation of bodipy-C_5-PC, a fluorescent analogue of miltefosine. The protein, which is a pump responsible for efflux and sequestration of compounds from the cell, has also been implicated in resistance to antimonials in *Leishmania* (reviewed in Ullman 1995). However, the fact that Miltefosine has been used successfully to treat antimonial-resistant cases of leishmaniasis (Sundar *et al.,* 1999) suggests resistance occurs via separate mechanisms. Recently it was shown that Miltefosine-resistant laboratory-derived *L. donovani* were deficient in uptake of the drug (Perez-Victoria *et al.,* 2003a). An aminophospholipid translocase transporter was subsequently shown to mediate Miltefosine influx and different point mutations in the gene encoding the translocase, *LDMT*, were responsible for conferring resistance (Perez-Victoria *et al.,* 2003b). Thirdly, as mentioned, the membrane-lipid composition and metabolism has been shown to be altered in resistant lines (Rakotomanga *et al.,* 2005). The authors suggest that interactions between Miltefosine and the cell membrane may be important for parasite susceptibility to the drug at higher concentrations.

Despite the high efficacy of Miltefosine in treating leishmaniasis and the benefits of its oral administration, the threat of drug-resistance developing in the field cannot be ignored. Moreover, the drug may not be suitable for treating leishmaniasis caused by some species of *Leishmania*: an *in vitro* study has shown a lack of sensitivity to Miltefosine of *L. braziliensis, L. mexicana* and *L. guyanensis* clinical isolates (Yardley *et al.,* 2005), while a cure rate of just 53% was achieved when the drug was used to treat cutaneous leishmaniasis (likely to be caused by *L. braziliensis*) in a clinical trial in Guatemala (Soto *et al.,* 2004). The efficacy of Miltefosine in treating HIV-co-infected patients has also been questioned (Berman *et al.,* 2006) after the majority of patients relapsed in one study (Sindermann *et al.,* 2004). Although Miltefosine is well-tolerated, it is not suitable for

treating pregnant females as it is teratogenic and must be administered with contraception to women of child-bearing age. These issues highlight the potential problems with Miltefosine, and suggest it is not necessarily the "wonder-drug" it has been hailed as.

1.3.3 Pentamidine

Pentamidine has toxic side effects but was originally extremely effective against infections of *Leishmania* and for several decades has been used as a second-line therapy for patients not responding to treatment with antimonials. However, pentamidine unresponsiveness has emerged (Giri, 1994) and in parts of India the cure-rate fell to less than 70% (Sundar, 2001a). Accordingly, its use as an antileishmanial drug has diminished. Together with other diamidine compounds, pentamidine is also used to treat types of pneumonia and sleeping sickness as it is active against both *T. brucei* and *Pneumocystis carinii*.

The characterisation of pentamidine-resistance *Leishmania* strains developed in the laboratory has contributed to the understanding of how the drug may act on the parasite. Pentamidine-resistant *L. donovani* and *L. amazonensis* had lower levels of putrescine but higher levels of ornithine and arginine compared to wild type strains and lower levels of the enzyme ornithine decarboxylase. Moreover, the affinity of spermidine synthase for pentamidine was decreased in resistant strains and the enzyme had a higher affinity for putrescine (Basselin *et al.*, 1997a). Pentamidine has also been found to inhibit arginine, putrescine and spermidine transport in *Leishmania* (Reguera *et al.*, 1994; Kandpal *et al.*, 1996). Pentamidine-mediated alterations in polyamine synthesis or uptake may therefore be responsible for parasites' susceptibility to the drug. Altered accumulation of pentamidine has been observed in drug-resistant *L. mexicana, L amazonensis* and *L. donovani* parasites (Basselin *et al.*, 1997b; Basselin *et al.*, 2002). Uptake of pentamidine is mediated by the P2 transporter in trypanosomes (reviewed by Bray *et al.*, 2003) but this is not the case in *Leishmania* and the route of entry into the parasites remains unclear. However, increased efflux rather than decreased influx of the drug is responsible for reduced accumulation of pentamidine in resistant *Leishmania* (Basselin *et al.*, 1997b). This has been attributed to resistant *L. mexicana* parasites not accumulating the drug in the mitochondrion as sensitive parasites do, therefore rendering the drug available for efflux (Basselin *et al.*, 2002). This is thought to be due to reduced uptake into the organelle and recently a P-glycoprotein-like translocase, PRP1, has been identified that may mediate transport of pentamidine into the mitochondrion; part of *PRP1* was deleted in drug-resistant parasites (Coelho *et al.*, 2003). Pentamidine is thought to adversely affect this organelle in *Leishmania*: treatment with the drug caused a decrease in the mitochondrial

membrane potential (Basselin and Robert-Gero 1998), disintegrated the organelle (Croft and Brazil 1982) and altered kDNA minicircle structure (Basselin *et al.*, 1998).

1.3.4 Amphotericin B preparations

Amphotericin B is a polyene antibiotic made by *Streptomyces* that interacts with sterols in plasma membranes, creating transmembrane channels which alter cells' permeability to cations, water and glucose and thus affecting the intracellular environment (Brajtburg and Bolard, 1996). Also an anti-fungal, it has a greater affinity for ergosterol (the predominant sterol in *Leishmania*) than cholesterol (the predominant sterol in mammalian cells) and therefore is more toxic to the parasite. However, dose-limiting renal toxicity in humans is a major problem and has resulted in the drug being a second line choice for treatment of leishmaniasis. In recent years, liposomal preparations of the drug have been developed in which the drug is delivered within a lipid bilayer (Ambisome) and these formulae are much less toxic to the host (Sundar *et al.*, 2002). However, the expense of Ambisome has resulted in it being unavailable in poor regions where leishmaniasis is endemic.

Fungal infections have been reported that display drug resistance when treated with amphotericin B (Kremery and Barnes, 2002). However, *Leishmania* resistance is not a problem in the field: reports of multiple relapses after treatment with amphotericin B and Ambisome in immuno-compromised patients are likely to be due to patient immune status rather than acquired parasite resistance (Durand *et al.*, 1998; Di Giorgio *et al.*, 1999). *Leishmania donovani* promastigotes resistant to amphotericin B have been created in the laboratory by selection after increasing drug pressure (Mbongo *et al.*, 1998). Analysis of these parasites revealed that amphotericin B uptake was decreased and efflux was increased, and that rather than the major sterol being the ergosterol lipid found in drug-sensitive parasites, an ergosterol precursor was present. However, the inability of these mutants to infect animals *in vivo* suggests that this lipid composition is not conducive to survival of *Leishmania* amastigotes and is therefore unlikely to be a problem in the field. The S- adenosyl-l-methionine-C_{24}-Δ-sterol-methyltransferase (SCMT) enzyme which mediates methylation of C-24 sterols was postulated to play a role in the phenotype and defective transcripts of SCMT were identified in amphotericin B-resistant parasites (Pourshafie *et al.*, 2004). More recently, laboratory-derived, Amphotericin B-resistant *L. mexicana* parasites have been created that are insensitive to the drug *in vitro* and *in vivo* (Al-Mohammed *et al.*, 2005). The parasites again had an altered sterol composition although they were infectious to animals, albeit causing attenuated disease symptoms. This may be cause for concern if such a situation occurs in the field.

1.3.5 Drugs not yet licensed: paromomycin, azoles and sitamaquine

Paromomycin (aminosidine) is another antibiotic which has antileishmanial activity. The lack of published studies on mechanisms of action and resistance is perhaps surprising, given that it is currently in phase III clinical trials and is likely to soon be available as mainstream treatment for visceral leishmaniasis. Paromomycin is also useful in treating cutaneous leishmaniasis (el-On *et al.*, 1992) although it is not as effective as antimonial treatment (Faghihi and Tavakoli-kia, 2003; Moosavi *et al.*, 2005). The mode of action of the drug in parasites has not been well characterised, although in bacteria the compound inhibits protein synthesis by binding ribosomal RNA (Schroeder *et al.*, 2000). Paromomycin has been shown to inhibit RNA and protein synthesis in *L. donovani* promastigotes and also affected lipid composition, membrane fluidity, and macromolecule uptake (Maarouf *et al.*, 1998). In a separate study, the drug was reported to affect the *Leishmania* mitochondria and respiration (Maarouf *et al.*, 1997). Parasites exhibiting drug-resistance to the compound have not yet been observed in the field, probably due to the limited use of paromomycin so far (Croft *et al.*, 2006). However, there are reports of drug-resistant parasites having been created in the laboratory (el-On *et al.*, 1991; Maarouf *et al.*, 1998) which retained their infectivity. The mechanism of resistance has not been defined, although a decrease in the uptake of the drug was observed (Maarouf *et al.*, 1998).

Cutaneous leishmaniasis has been treated successfully with itraconazole (Consigli *et al.*, 2006) and ketoconazole (Salmanpour *et al.*, 2001), oral formulations in clinical trials which inhibit ergosterol synthesis in the parasite. In addition, azoles may be useful in treating mucocutaneous leishmaniasis (Amato *et al.*, 2000). However, the efficacy of these compounds is uncertain and one study showed that itraconazole was no better than the placebo in treating cutaneous disease caused by *L. major* (Nassiri-Kashani *et al.*, 2005). No acquired resistance to azoles has been reported in the field although over-expression in *L. major* of squalene synthase, which has a role in mediating ergosterol biosynthesis, resulted in reduced sensitivity to itraconazole (Cotrim *et al.*, 1999).

Sitamaquine (WR6026) is a second orally active drug which is currently in clinical trials for efficacy in treating visceral leishmaniasis (Yeates, 2002). Although the drug has the advantage of oral administration, the cure-rates of visceral leishmaniasis in a phase II clinical trial conducted in Brazil were unimpressive (Dietze *et al.*, 2001) and toxic side-effects were observed. Sitamaquine was more effective at treating leishmaniasis caused by

L. donovani in India and Kenya (Wasunna *et al.*, 2005). The mode of action is unknown and no studies have been published into sitamaquine-resistance in *Leishmania*.

1.4 Pentavalent antimonials

As mentioned, all forms of leishmaniasis are usually treated primarily with pentavalent antimonials. Herein these will be referred to as Sb(V), and when necessary the different drug compounds available will be referred to as SSG (sodium stibogluconate) and MGA (meglumine antimoniate). Meanwhile trivalent antimonials will be referred to as Sb(III) unless otherwise stated. Throughout this section, mechanisms which may confer resistance to antimonials are also discussed as resistance to antimonials is an increasing problem in the field (Sundar, 2001a).

Despite their prolonged use, many aspects of antimonial function – including uptake, metabolism, detoxification and mechanism of action of the drug – remain uncertain. The relatively low level of antimony in the environment has resulted in minimal contamination with this metal and subsequently antimonial poisoning is not a problem for humans. Therefore, little research has been carried out on the toxic effects of antimony in this aspect as well. On the other hand, arsenic - a metalloid (or semimetal) element similar to antimony - has been much more extensively characterised (probably due to its toxic effects often being seen in humans due to its abundance in the ground and contamination of drinking water) and more is known about its toxicology. Accordingly, it is often necessary to consider research carried out on arsenical compounds when analysing how antimonial compounds behave (Gebel 1997). The pentavalent form is known as arsenate (As(V)) while the trivalent form is arsenite (As(III)).

Promastigotes are not susceptible to Sb(V) although they are to Sb(III) (Ephros *et al.*, 1999). As discussed in detail in section 1.4.3, it is generally accepted that Sb(V) is effectively a pro-drug and that reduction to a trivalent form is necessary for antileishmanial toxicity, although whether this reaction is carried out by the host cell or the parasite remains controversial. Accordingly, investigations into both Sb(V) and Sb(III) have been evaluated here.

The presence of the preservative chlorocresol in Sb(V) preparations has hindered research into the drugs, as chlorocresol itself has antileishmanial activity (Roberts and Rainey 1993). Specifically, studies into drug-resistance have been affected: unlike Sb(V), chlorocresol is toxic *in vitro* to promastigotes and therefore laboratory-derived Sb(V)-

resistant *Leishmania* strains may actually be resistant to the preservative rather than the drug (Ephros *et al.*, 1997). Accordingly, whether chlorocresol was present in Sb(V) preparations has been considered where possible when reviewing literature on antimonials.

1.4.1 Synthesis and structures

Both formulations of the pentavalent antimonials are synthesised with chelating agents which improve the solubility of the drugs. SSG is produced by reacting pentavalent antimony with gluconic acid. The result is a complex mixture of antimonial and carbohydrate species ranging in size and structure (Berman and Grogl, 1988). The mixture is assayed for its pentavalent antimony content and the drug is prepared using a standard amount. There has been some debate over whether the quality of each batch remains constant (Jackson *et al.*, 1990) but fractionation of SSG by ion-exchange chromatography revealed that each fraction had similar activity against *L. panamensis* amastigotes. The antileishmanial activity of the drug is, however, probably due to several compounds in the preparation (Roberts and Rainey, 1993). Despite this, the main component of the polymeric structure has been proposed (figure 1.3A). The second antileishmanial form of pentavalent antimony is MGA, which is made by reacting pentavalent antimony with *N*-methyl-D-glucamine. Analysis of the drug revealed that it is composed of a number of compounds, although a major component was identified that had a molecular mass of 507 atomic mass units, the structure of which was proposed by the authors (figure 1.3B) (Roberts *et al.*, 1998). Despite the differing structures of the drugs and reports of Pentostam being used to treat Glucantime-resistant cases of leishmaniasis (Moreira *et al.*, 1992), few differences have been reported in their efficacy and treatment outcomes are similar.

1.4.2 Uptake of antimonials

How Sb(V) enters *Leishmania* is not well characterised. Studies using radioactive sodium stibogluconate showed that amastigotes accumulate the drug more quickly and to a higher concentration than either promastigotes or macrophages (Berman *et al.*, 1987). The authors suggest that uptake of the compound could be due to non-enzyme mediated diffusion of the drug across the membrane due to macromolecule binding within the parasite. The observation that amastigotes accumulate approximately three times the amount of Sb(V) than promastigotes and macrophages could account for both the parasite- and stage-specificity of the drug. Using a more recently developed technique known as inductive coupled plasma mass spectrometry, similar results were obtained in *L. tarentolae*, *L.*

infantum and *L. donovani*: amastigotes accumulated several times the amount of Sb(V) than promastigotes (Brochu *et al.*, 2003). It was also observed in both studies that there was no competition between Sb(III) and Sb(V) for entry into the parasites, indicating that the drugs enter the cells by different mechanisms.

The uptake mechanism of Sb(V) has not been elucidated in other organisms (Tamas and Wysocki, 2001) although limited research has been carried out on pentavalent arsenicals. As(V) has a similar structure to inorganic phosphate and accordingly, they enter bacterial cells via the same transporters. In *Escherichia coli*, this is an ABC type ATPase complex formed by four separate proteins (reviewed in Gatti *et al.*, 2000). Mutated phosphate transporters have been linked to increased arsenate tolerance in *E. coli* (Willsky and Malamy, 1980). In addition, in human cell lines, arsenate uptake is inhibited by phosphate (Huang and Lee 1996).

An aquaglycerolporin (AQP1) has been recently identified in *Leishmania* which mediates the uptake of Sb(III) (Gourbal *et al.*, 2004). When AQP1 was over-expressed in *Leishmania* species, the parasites became hypersensitive to Sb(III) and expression of the protein in resistant isolates induced sensitivity. Interestingly, over-expression of AQP1 in a Sb(V)-resistant strain of *L. donovani* conferred sensitivity to Sb(V) as well as Sb(III), implying that reduction of the pentavalent drug to Sb(III) occurs, at least partially, in the macrophage (Gourbal *et al.*, 2004). The expression of *AQP1* was also found to be decreased in Sb(V) resistant field isolates (Decuypere *et al.*, 2005). AQP1 is a member of the family of aquaporins -- channels which small, neutral solutes such as glycerol can pass through – and similar proteins have been found in other organisms: Fps1p in *S. cerevisiae* (Wysocki *et al.*, 2001) and GlpF in *E. coli* (Sanders *et al.*, 1997) mediate Sb(III) uptake.

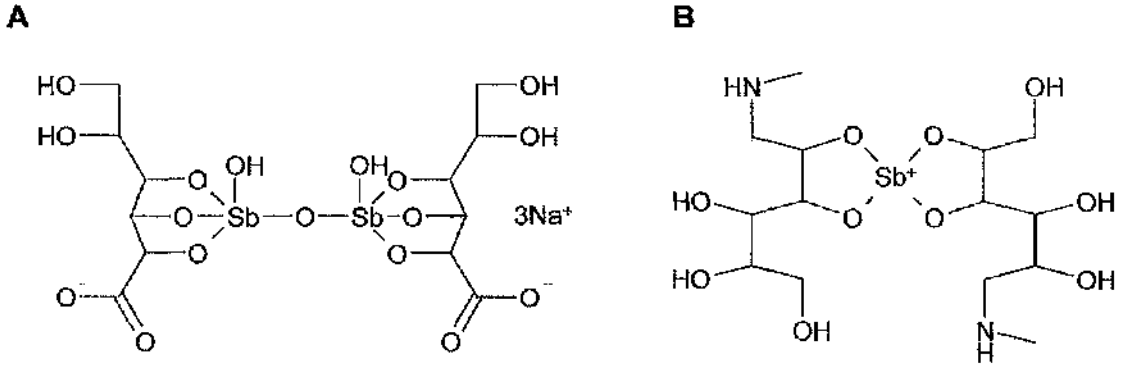


Figure 1.3: Structures of pentavalent antimonial drugs used to treat leishmaniasis. A, sodium stibogluconate. Image redrawn from previously reported image Croft *et al.*, 2006. B, meglumine antimoniate. Image redrawn from previously published image Roberts *et al.*, 1998.

1.4.3 Antimonial metabolism

1.4.3.1 Reduction of pentavalent antimonials

Although antimonials are administered in a pentavalent form, it is hypothesised that the drugs are reduced to a trivalent form, which is more toxic to *Leishmania* (Roberts *et al.*, 1995; Sereno and Lemesre, 1997). It is not known why reduction occurs, although in other organisms it is the first stage in the detoxification of metalloid compounds: this will be discussed in section 1.4.5. Since the extra-cellular promastigote life-cycle stage of the parasite is not susceptible to Sb(V), two hypotheses have arisen: that reduction takes place in the host cell and that therefore only amastigotes are exposed to the toxic trivalent form (Sereno *et al.*, 1998) or that reduction to the trivalent form is performed by the amastigotes parasites themselves in a stage-specific manner (Shaked-Mishan *et al.*, 2001). Supporters of the concept that reduction is performed by the host point to the fact that mammalian *Leishmania* hosts are indeed able to reduce Sb(V): when pentavalent drugs were administered, Sb(III) was detected in both hamsters (Lugo de Yarbuh *et al.*, 1994) and humans (Goodwin and Page, 1943). It was recently shown that over-expression of a Sb(III) transporter (AQP1) in *Leishmania* conferred sensitivity of the parasites to Sb(V) when incubated in macrophages (Gourbal *et al.*, 2004). This suggests that at least a proportion of the pentavalent drug is metabolised to Sb(III) by the macrophage and that the subsequent uptake of the trivalent form accounts for the increased susceptibility to the drug. However, further investigation is required to show that that is indeed the case; in one recently published study Sb(V), unlike Sb(III), was not found to be toxic to macrophages and hence it was concluded that they are unable to mediate reduction of the drug (Wyllie and Fairlamb 2006). It has been shown that unlike intracellular amastigotes, axenically cultured *L. infantum* amastigotes were not susceptible to Sb(V), suggesting that macrophages mediated Sb(V) reduction (Sereno *et al.*, 1998). However, this observation has been challenged as several groups have shown that both *L. mexicana* and *L. donovani* axenic amastigotes are indeed sensitive to Sb(V) (Coombs *et al.*, 1983; Callahan *et al.*, 1997; Ephros *et al.*, 1999; Goyard *et al.*, 2003). Furthermore, it was recently found that the effect of Sb(III) on *L. donovani* promastigotes – the simultaneous efflux of glutathione and trypanothione and the accumulation of oxidised thiols due to inhibition of trypanothione reductase – was the same as the effect of Sb(V) on *L. donovani* axenic amastigotes (Wyllie *et al.*, 2004). These data imply that the macrophage is not required for the antileishmanial activity of Sb(V) and that therefore the parasites themselves can, at least in part, reduce the drugs. Compellingly, it has also been shown that *L. donovani* parasites are indeed able to reduce antimonials and that amastigotes reduce Sb(V) to Sb(III) to a much greater degree

greater degree than promastigotes (Shaked-Mishan *et al.*, 2001). Furthermore, the reducing activity correlated with Sb(V) sensitivity as laboratory-derived, Sb(V)-resistant *L. donovani* amastigotes were unable to reduce the pentavalent compound.

The mechanism of Sb(V) reduction has not been fully elucidated. It is possible that the reaction is enzymatically controlled (Shaked-Mishan *et al.*, 2001) but it has recently been shown that the reduction of pentavalent antimony can occur spontaneously *in vitro* upon reaction with various thiols including glutathione, trypanothione and cysteine (Frezard *et al.*, 2001; Ferreira Cdos *et al.*, 2003; Yan *et al.*, 2003a; Yan *et al.*, 2003b). An acidic environment was required for these non-enzymatic reactions to occur, similar to that of the parasitophorous vacuoles which *Leishmania* amastigotes reside in *in vivo*. It is therefore possible that any reduction of Sb(V) that does occur in the macrophage is mediated non-enzymatically in this way. It was observed that the rate of Sb(V) reduction was greater upon reaction with the parasite-specific thiol trypanothione than with the ubiquitous glutathione (Ferreira Cdos *et al.*, 2003) and, in theory, this could account for the greater toxicity of Sb(V) towards the parasites than the host cell. However it does not explain the stage-specificity of the drug as the levels of both trypanothione and glutathione are higher in promastigotes than in Sb(V)-sensitive amastigotes (Ariyanayagam and Fairlamb, 2001). It is also doubtful whether such reactions could proceed in the neutral intracellular parasite environment. Accordingly, enzymes that may have the ability to mediate the reduction of Sb(V) are discussed in the following section.

1.4.3.2 Arsenate reductases

Several enzymes capable of reducing arsenate have been characterised from various prokaryotic and eukaryotic species. The number of categories of proteins known to mediate this reaction has doubled from three to six in recent years, reflecting the relative lack of knowledge in the area, particularly in higher eukaryotic metalloid metabolism. It is possible that some or all of these enzymes are also able to reduce pentavalent antimonials although there are no reports of this activity being sought. Here, enzymes currently known to reduce arsenate – which can therefore be thought of as potential Sb(V) reductases – are described. The proteins are summarised in table 1.2.

Enzyme Name	Organisms found in	*In vivo* evidence of role in arsenate metabolism?
ArsC (R773 plasmid)	The gram-negative bacteria *E. coli*	Yes
ArsC (pI258 plasmid)	The gram-positive bacteria *Subtilis aureus* and *Bacillus subtilis.*	Yes
Acr2p	Yeast and plants: *Saccharomyces cerevisiae, Arabidopsis thaliana, Pteris vittata.*	Yes
Purine Nucleoside Phosphorylase (PNP)	Ubiquitous, but As(V) reducing activity characterised in mammalian enzymes (human, rat, calf, mouse, rabbit, hamster, guinea pig).	No
glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Ubiquitous, but As(V) reducing activity characterised in mammalian enzymes (human and rat).	No
Omega GST (oGST)	Variety of organisms including human, pig, *S. mansoni, S. cerevisiae* and *Drosophila melanogaster*. As(V) reducing activity found in human and S. cerevisiae enzymes.	No

Table 1.2: Arsenate reductases. All enzymes known to possess arsenate reductase activity, *in vivo* and *in vitro* (as indicated), are listed. Bacterial enzymes are in purple, the yeast and plant Acr2p is shown in blue, and mammalian enzymes are in red.

There are three main classes of microbial arsenate reductases known to exist which, although they perform the same function, are completely different enzymes (Mukhopadhyay and Rosen, 2002). The first of these, ArsC, is encoded for on the R773 plasmid of *E. coli* and by part of the *ars* operon. The entire operon confers resistance to Sb(III) when over-expressed (Carlin *et al.,* 1995), suggesting both compounds can be used as substrates by the encoded proteins. Unfortunately the authors did not assay susceptibility to Sb(V) so whether ArsC can reduce pentavalent antimonial compounds is unknown. ArsC uses reduced glutathione (GSH) to convert arsenate to arsenite via the following reaction:

$$H_3AsO_4 + 2GSH \longrightarrow H_3AsO_3 + GS\text{-}SG + 2H_2O.$$

ArsC becomes oxidised when it catalyses the reaction and is reduced by *E. coli* glutaredoxin 2 (Shi *et al.,* 1999). In turn, the oxidised glutaredoxin 2 is reduced by GSH which is converted to GSSG which in turn is reduced by glutathione reductase, with NADPH serving as the source of reducing potential (Liu and Rosen, 1997). The crystal structure of the complex has been elucidated and shows that the arsenate binds to the anion

site of ArsC, which is made up of Arg60, Arg94, Arg107, and then forms a covalent arsenate thioester with Cys12. After being reduced by glutathione and glutaredoxin, the Cys12-S-arsenite intermediate hydrolyses and the arsenite is released (Martin *et al.*, 2001). ArsC has low structural similarity to glutaredoxin and glutathione S-transferase.

Confusingly, the second class of arsenate reductases is also called ArsC, despite being unrelated to the type of enzymes described above. Found in several prokaryotic species, the protein is best characterised in *Staphylococcus aureus* and *Bacillus subtilis* and the crystal structures have been solved (Bennett *et al.*, 2001; Zegers *et al.*, 2001). Like ArsC arsenate reductase from *E. coli, S. aureus ArsC* is part of an operon encoded for on a plasmid, called pI258. The reaction is dependant on thioredoxin and thioredoxin reductase (as opposed to the former class which relies on glutaredoxin and glutaredoxin reductase) (Ji and Silver, 1992; Ji *et al.*, 1994). The catalysis is proposed to begin with Cys82 attacking Cys10 and forming a disulphide bond. Cys10 donates its electron pair to the arsenate, which reduces it to arsenite. Cys89 attacks Cys82 to form a disulphide and regeneration of the Cys10 thiolate occurs. Finally, ArsC is restored by thioredoxin and resumes its conformation. Similar mechanisms are present in the *S. aureus* and *B. Subtilis* proteins (Bennett *et al.*, 2001; Zegers *et al.*, 2001). As well as reducing arsenate, the pI258 and the *B. subtilis* protein also function as tyrosine phosphatases and the crystal structures revealed that they have structural similarity to low molecular weight tyrosine phosphatases. The first step in the catalysis of arsenate reduction is analogous to that of tyrosine dephosphorylation (Bennett *et al.*, 2001; Zegers *et al.*, 2001).

The first eukaryotic arsenate reductase, Acr2p, was found in the yeast *Saccharomyces cerevisiae* almost a decade ago (Bobrowicz *et al.*, 1997; Mukhopadhyay and Rosen, 1998). More recently, a similar enzyme has been identified in plants and *A. thaliana Acr2p* gene knockouts lacked the ability to reduce arsenate and accumulated the pentavalent metalloid (Duan *et al.*, 2005; Dhankher *et al.*, 2006). The *S. cerevisiae* enzyme has been characterised (Mukhopadhyay *et al.*, 2000; Mukhopadhyay and Rosen, 2001) although not to the extent of the previous two classes of prokaryotic arsenate reductases. Despite the fact it is not related to either of these two classes, Acr2p is related to protein tyrosine phosphatases albeit from a different class than that of the pI258 ArsC-related low molecular weight tyrosine phosphatases. One phosphatase of this class is CDC25a, which is involved in the human cell cycle and contains the $HC(X)_5R$ motif in its active site that is also present in the *S. cerevisiae* Acr2p. When the Acr2p site is mutated, the enzyme no longer has arsenate reductase activity *in vitro* (Mukhopadhyay and Rosen, 2001) suggesting they are likely to have common active sites. Conversely, Acr2p does not have

tyrosine phosphatase activity and lacks the phosphate binding GXGXXG motif of CDC25a. However, when this site is introduced by the addition of glycines into Acr2p, the enzyme gains this activity (Mukhopadhyay *et al.*, 2003). It is hypothesised that this class of arsenate reductase evolved from protein-tyrosine phosphatases. Like *E. coli* R773 ArsC, the enzyme activity is glutaredoxin- and glutathione reductase-dependant (Mukhopadhyay *et al.*, 2000).

Until recently, the identity of an enzyme responsible for the reduction of arsenate in higher eukaryotes has remained elusive. Although the mechanism is still largely unknown, the activity has recently been attributed to purine nucleoside phosphorylase (PNP), an enzyme that normally uses phosphate to cleave purine nucleosides (inosine or guanosine) into bases (hypoxanthine or guanine) and ribose-1-phosphate. In the presence of inosine and the endogenous dithiol, dihydrolipoic acid (DHLP), or a synthetic equivalent such as dithiothreitol (DTT), calf spleen PNP was shown to reduce arsenate (Radabaugh *et al.*, 2002). The work was supported by observations that specific PNP inhibitors also inhibit rat liver cytosolic arsenate reductase activity, as do phosphate, the natural substrate of the enzyme, and guanine and hypoxanthine, the natural products of PNP which promote the reverse reaction (Gregus and Nemeti, 2002). Despite encouraging results showing that the rate of arsenate reduction in erythrocytes could be stimulated by the addition of exogenous DTT and inosine or guanosine, inhibition of PNP in both erythrocytes and rats with blocked excretory routes (to prevent clearance of the compounds) did not alter the base rate of arsenate reductase activity or the relative As(V) and As(III) levels (Nemeti *et al.*, 2003). This suggests that an alternative mammalian enzyme may be responsible for reducing arsenate *in vivo*.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is the second mammalian enzyme purported to have a role in arsenate reduction (Gregus and Nemeti, 2005). Clearly the primary function of this enzyme is not to reduce metalloids: GAPDH catalyses the oxidative phosphorylation of D-glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate which is a step in the glycolysis pathway. However, purified GAPDH reduced As(V) in the presence of GSH and NAD. Koningic acid (KA), a specific inhibitor of GAPDH, abolished arsenate reductase activity in erythrocytes (Gregus and Nemeti 2005). Furthermore, administration of (S)-alpha-cholorhydrin (ACH), which forms a GAPDH inhibitory metabolite, resulted in a significant reduction in arsenate reductase activity in rat liver (Nemeti *et al.*, 2005). However KA failed to prevent arsenate reduction in rat liver cytosolic extracts and rats treated with ACH were capable of reducing arsenate despite the effect observed in the liver. These experiments were carried out with PNP inhibitors also

present: the fact that arsenate reduction still occurs when both GAPDH and PNP are inhibited indicates that another protein is capable of mediating the reaction. Further analysis is clearly required to determine whether the two enzymes do have an *in vivo* role in mammalian arsenate reduction.

The sixth type of arsenate reductase, omega glutathione S-transferase (oGST), was first characterised as a methylated arsenical reductase (see section 1.4.3.3). Recently it has been reported that the protein is also able to reduce inorganic arsenate *in vitro* (Aposhian *et al.*, 2004) although the details of this have not yet been published. Human oGST was the first to be characterised (Board *et al.*, 2000) and oGSTs from a range of organisms including pig (Rouimi *et al.*, 2001), *Drosophila melanogaster* (Kim *et al.*, 2006) *Schistosoma mansoni* (Girardini *et al.*, 2002), and *S. cerevisiae* (Garcera *et al.*, 2006) have since been studied. The oGSTs have a molecular mass of around 27.5 kD and have 20 residue N-terminal extensions not present in other classes of GSTs. They are highly expressed in human liver, macrophage, glial and endocrine cells and were found to be localised in the nucleus (Yin *et al.*, 2001). However, the pig oGST is reportedly cytosolic (Rouimi *et al.*, 2001). In *S. mansoni*, the protein was detected in all life cycle stages but expression was highest in the sporocysts (the parasitic stage of the intermediate host) and adult worms (parasitic stage in human) (Girardini *et al.*, 2002). All of the known oGSTs share an active site motif of C-P-Y/F-A/V/S which resembles those of the monothiol glutaredoxins; moreover, oGST shares structural similarity with *E. coli* glutaredoxin 2 (Xia *et al.*, 2001). Despite being classed as a GST, the *in vitro* activities of all oGSTs characterised so far correlate more closely with glutaredoxins: the proteins exhibit non-detectable or very low GSH and 1-chloro-2,4-dinitrobenzene (CDNB) conjugating activity – the standard GST activity – and higher thioltransferase and dehydroascorbate activities – characteristic of glutaredoxins (Board *et al.*, 2000; Rouimi *et al.*, 2001; Garcera *et al.*, 2006). However, a second human oGST displays high GSH and CDNB conjugating activity (Wang *et al.*, 2005). The role of oGST in metalloid reduction *in vivo* has not been unequivocally demonstrated, although naturally-occurring polymorphisms in *oGST* have been linked to arsenic-susceptibility in mammals (Schmuck *et al.*, 2005) and alterations in excreted arsenical profiles in humans (Marnell *et al.*, 2003).

1.4.3.3 Biomethylation

As described, the reduction of pentavalent arsenicals and antimonials creates more toxic trivalent forms of the metalloids. However, further metabolism of these compounds is possible and the *in vivo* transformation of arsenicals and antimonials from inorganic to

organic molecules via the addition of methyl groups (biomethylation) is well documented. The biomethylation pathway involves alternating reduction and oxidative methylation of arsenate (figure 1.4). A mammalian enzyme originally known as Cyt19 but more recently referred to as AS3MT is responsible for the methyltransferase activity (Lin *et al.*, 2002; Wood *et al.*, 2006). Although the recombinant protein is not thought to directly reduce pentavalent, methylated arsenicals, the reduction step was performed when either exogenous (DTT or tris(2-carboxyethyl)phosphine hydrochloride (TCEP)) or endogenous (thioredoxin, thioredoxin reductase and NADPH) reductants were present (Thomas *et al.*, 2004). However, at least one enzyme is capable of mediating reduction of the methylated arsenicals: oGST displays this activity *in vitro* (Zakharyan *et al.*, 2001), along with the pentavalent inorganic arsenate reductase activity described in section 1.4.3.2.

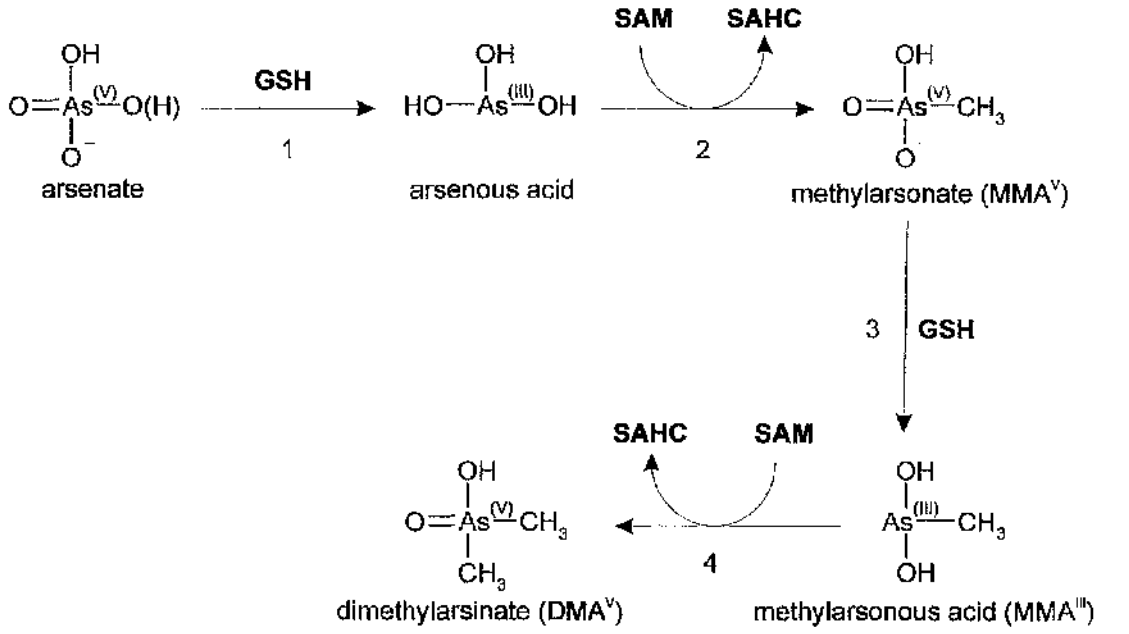


Figure 1.4: Putative pathway of biotransformation of inorganic arsenate. 1, arsenate reductase (ArsC (R773), ArsC (PI258), Acr2p, PNP, GAPDH, oGST); 2, arsenite methyltransferase (AS3MT); 3, MMA(V) reductase (oGST) 4, MMA(V) transferase. GSH, glutathione; SAM, *S*-adenosylmethionine; SAHC, *S*-adenosylhomocysteine. Figure redrawn from previously published image Zakharyan *et al.*, 2001.

Although many studies of biomethylation have addressed the methylation of arsenicals in mammalian systems (Zakharyan *et al.*, 1999), a similar process is known to occur in various microbes with antimonial species (reviewed in Bentley and Chasteen, 2002). However, whether the mechanism is similar for antimonials and arsenicals and if similar

enzymes mediate the process is unknown. The purpose of the biomethylation pathway is not clear. Originally thought to be a detoxification pathway, this hypothesis has been discredited due to the greater toxicity of methylated, trivalent species than inorganic arsenite (reviewed in Thomas *et al.*, 2001). The effects of methylated arsenicals include the inhibition of thioredoxin and glutathione reductase (Styblo *et al.*, 1997; Lin *et al.*, 1999), genotoxicity (Mass *et al.*, 2001) and cytotoxicity (Petrick *et al.*, 2000; Styblo *et al.*, 2000).

The occurrence of biomethylation of antimonials may be important when investigating how antimonial drugs are metabolised by *Leishmania*. It is not known whether the parasites are capable of performing these reactions, or whether after reduction of the drug to the trivalent form no further metabolism takes place. However, the new data emerging concerning the toxicology of biomethylated intermediates and products suggests that if *Leishmania* can biomethylate antimonials, there could be important implications for how the drugs are lethal to the parasites.

1.4.3.4 Trivalent metalloid oxidation

Several species of bacteria capable of oxidising As(III) to As(V) – effectively the reverse reaction to that mediated by the arsenate reductases – have been identified including *Alcaligenes faecalis* (Philips and Taylor 1976), *Thermus aquaticus* (Gihring *et al.*, 2001) and *Agrobacterium tumefaciens* (Kashyap *et al.*, 2006). Two enzymes which mediate oxidation of the metalloid are the aoxA and aoxB proteins originally found in *Alcaligenes faecalis* (Anderson *et al.*, 1992). The characteristics of these proteins, together with bacteria and archea which possess genes likely to encode similar enzymes, are reviewed in Silver and Phung, 2005. The two genes, which are part of a larger "arsenic detoxification" operon, encode a large subunit which contains a molybdenum-binding site and a smaller iron-sulphur subunit which contains a Rieske domain. Expression of the operon, which contains elements thought to regulate transcription of the encoded proteins, is upregulated in response to exposure to As(III) (Kashyap *et al.*, 2006). When the sequence of either of the genes that encode aoxA or aoxB was disrupted in *Cenibacterium arsenoxidans*, the arsenite oxidase activity usually displayed by the bacteria was abolished (Muller *et al.*, 2003a). Reports of bacteria able to oxidise Sb(III) also exist (Lialikova 1974), although whether the proteins that mediate arsenate oxidation can also oxidise Sb(III) is unknown.

It was recently reported that As(III) could be oxidised to As(V) non-enzymatically upon reaction with hydrogen peroxide (H_2O_2) (Aposhian *et al.*, 2003) and this has now also been shown to occur with Sb(III) (Quentel *et al.*, 2004). It has been postulated that arsenite

could be metabolised in such a way *in vivo* (Aposhian *et al.*, 2003) although this may not be the case as an alkaline environment was required (Quentel *et al.*, 2004). It is unknown whether non-enzymatic oxidation of trivalent metalloids can occur *in vivo* or whether enzymatic oxidation takes place in eukaryotic species. If either of these mechanisms are found to persist it would radically alter the field of metalloid metabolism in eukaryotes, which has so far been perceived as unidirectional (figure 1.4).

1.4.3.5 Arsenate metabolising-like enzymes in the trypanosomatids

Of all the different enzymes known to mediate metalloid metabolism in different organisms, homologues of only two – Acr2p and oGST – have been characterised in trypanosomatids: *Leishmania* Acr2p and the oGST-like Tc52 of *T. cruzi*. In addition, an oGST-like enzyme, TDR1, has been identified in *Leishmania*, the characterisation of which is described in this thesis.

Leishmania major Acr2p (LmACR2) has only recently been described (Zhou *et al.*, 2004). The purified protein was found to reduce both As(V) and Sb(V) to trivalent species *in vitro*. It was observed that the enzyme has a low specific activity when used in conjunction with glutaredoxin (required to keep LmACR2 reduced) and this observation has lead to its physiological relevance being questioned (Croft *et al.*, 2006). However, Zhou *et al.*, 2004 propose that this may be due to tryparedoxin, a parasite-specific equivalent of glutaredoxin, being the enzyme that reduces LmACR2 *in vivo*. When the *L. major* gene was expressed in As(V)-sensitive *E. coli*, arsenate resistance was conferred. This was thought to be due to the fact that reduction is necessary for the detoxification of metalloids in bacteria. Moreover, when LmACR2 was over-expressed in *L. infantum* the transgenic parasites displayed slightly increased sensitivity to sodium stibogluconate. The existence of LmACR2 was reported during the investigation reported in this thesis: its relevance is further discussed in chapter two.

The *T. cruzi* protein Tc52 (so called because of the species it is found in and its size, 52 kD) is the only protein characterised from a trypanosomatid that shares significant amino acid identity with any GST (Schoneck *et al.*, 1994) and is most similar to the omega GSTs. However, the protein has not been investigated in relation to metalloid susceptibility and so whether it shares the arsenate-reducing *in vitro* activity of oGST is unknown. Like oGST, Tc52 reportedly has no CDNB conjugating activity (Moutiez *et al.*,, 1995), but does possess dehydroascorbate reductase and thioltransferase activities which are characteristic of glutaredoxins (Moutiez *et al.*, 1997). These activities were strictly dependant on GSH

and Tc52 was unable to use the parasite-specific thiol trypanothione ($T(SH)_2$) as a hydrogen donor. In addition, Tc52 was found to enzymatically reduce oxidised GSH – glutathione disulphide (GSSG) – using $T(SH)_2$ as the source of reducing potential (Schoneck *et al.*, 1994; Moutiez *et al.*, 1995; Moutiez *et al.*, 1997). Trypanosomatids do not possess the glutathione reductase enzyme which reduces GSSG in other systems, and instead contain trypanothione reductase which regenerates oxidised $T(SH)_2$. Despite the similarities in activities between the two proteins, Tc52 is approximately 52 kD in size: double the size of omega GST. Analysis of the sequence showed that Tc52 consists of two similar halves which may have arisen from a gene duplication event (Schoneck *et al.*, 1994), and which both share sequence identity with oGSTs. However, the active site motifs in the N- and C- terminal halves of Tc52 are thought to be CPFC and SPFS, both of which differ from the equivalent oGST motifs (section 1.4.3.2). However, the C-terminal active site sequence was CPFS in *T. marinkellei*, a South American zoonotic parasite closely related to *T. cruzi* (Oury *et al.*, 2005); this motif is more similar to that of oGST.

Tc52 is most highly expressed in the epimastigote and amastigote life cycle stages and has been localised to cytoplasmic organelles at the posterior end of the parasites, thought to be reservosomes (Ouaissi *et al.*, 1995b). However, the protein has also been shown to be released into the culture media when the parasites are growing (Schoneck *et al.*, 1994) and microscopic analysis of *T. cruzi*-infected heart sections showed that Tc52 localised to the parasite surface and aggregated in the host cell (Garzon *et al.*, 2003). Secretion of Tc52 from parasites correlates with another observed function of the protein: modulation of the host immune response. Tc52 has been shown to induce various immune responses including the inhibition of T-cell proliferation in which a C-terminal region is thought to be responsible (Borges *et al.*, 2003), activation of macrophages characterised by increased expression of nitric oxide synthase and various chemokines (Fernandez-Gomez *et al.*, 1998) and dendritic cell maturation and chemokine expression upon binding to these cells (Ouaissi *et al.*, 2002). The immuno-regulatory role of Tc52 has also led to the protein being proposed as a vaccine candidate: immunised mice infected with *T. cruzi* parasites had significantly reduced mortality and parasitemia compared to non-immunised mice (Ouaissi *et al.*, 2002).

Allaoui *et al.*, 1999 attempted to create *T. cruzi* Tc52 null mutant parasites, although, because it proved impossible to knock out both alleles of the gene, the protein is thought to be essential. Parasites with only one copy of *Tc52* remaining and a corresponding reduced level of Tc52, exhibited an impaired ability to undergo metacyclogenesis (Allaoui *et al.*, 1999). Moreover, the intracellular trypomastigote forms did not proliferate as well as wild

type parasites when incubated in macrophages. This effect was also apparent *in vivo*: parasitemia was lower in mice infected with the Tc52 single-copy parasites than with wild type parasites (Garzon *et al.*, 2003). However, this observation has not yet been correlated with altered changes in the immune response of the host.

1.4.4 Modes of actions of antimonials

Although antimonials have been used for more than six decades to treat leishmaniasis, the mechanism of how they are toxic to cells remains unclear. Because toxicity of the drugs is thought to be mediated by reduction from the pentavalent to the trivalent from, it may be useful to consider the effect of both forms of the drug on the parasite and accordingly both are discussed here.

L. infantum amastigotes exposed to the trivalent antimonial potassium antimonial tartrate (KAT) undergo a series of cellular changes including DNA fragmentation that result in apoptosis (Sereno *et al.*, 2001) although the events that precede this remained ambiguous. Recently it was reported that treatment of intracellular *L. donovani* amastigotes with KAT resulted in parasite death characterised by DNA fragmentation and extrusion of phosphatidylserine (Sudhandiran and Shaha, 2003). High levels of reactive oxygen species (ROS) were produced before cell death but in the presence of an antioxidant, levels of ROS decreased and parasite survival increased. Following ROS production, a reduced parasite mitochondrial membrane potential and an elevated Ca^{2+} level was also observed, which did not occur when the cells were treated with an antioxidant alongside KAT. Flufenamic acid, a non-selective cation channel blocker, also inhibited these effects. Together, these results imply that oxidative stress caused by treatment with KAT causes Ca^{2+} influx through non-selective cation channels, and that this directly or indirectly leads to apoptosis (Sudhandiran and Shaha, 2003).

An additional observation regarding the activity of antimony is that Sb(V) (MGA and SSG) is capable of stabilising DNA-protein complexes in *Leishmania* promastigotes (Lucumi *et al.*, 1998) and inhibits the relaxation of supercoiled DNA (Chakraborty and Majumder, 1988). These observations are associated with the inhibition of the enzyme topoisomerase I which is involved in breaking and rejoining DNA. Furthermore, much higher concentrations of meglumine antimoniate were required to induce the stabilisation of DNA-protein complexes in Sb(V)-resistant *L. (viannia) panamensis* parasites than in wild type parasites (Lucumi *et al.*, 1998). However, whether *Leishmania* topoisomerase I is indeed an antimonial target is unclear as it was recently shown that Sb(V) but not Sb(III)

inhibited the enzyme in *L. donovani* promastigotes (Walker and Saravia, 2004). However, given that the *Leishmania* topoisomerase I was inhibited to a greater extent than the human enzyme by Sb(V), the enzyme may yet prove to be a drug target.

Enzymes from other organisms purported to be inhibited by antimonials include protein tyrosine phosphatase in murine hemopoietic cells (Pathak and Yi, 2001), unspecified glutathione S-transferases in human erythrocytes (Poon and Chu, 2000) and *S. mansoni* phosphofructokinase (Su *et al.*, 1996). In addition, it was shown that at least six proteins ranging in size from 14-68 kD were drug targets in *L. mexicana* amastigotes treated with radioactive SSG (Berman and Grogl, 1988). However, the identity of these proteins has not been elucidated. Glycolysis has also been proposed as a target of Sb(V) (Berman *et al.*, 1985) but again, clarification of how this occurs has not been forthcoming.

However, the most convincing evidence for how antimonials exert their effect on *Leishmania* concerns the modulation of the redox environment of the parasite. *L. donovani* trypanothione reductase has been shown to be inhibited in vitro by Sb(III) (Cunningham and Fairlamb, 1995). As mentioned, this enzyme regenerates oxidised $T(SH)_2$, the thiol found only in trypanosomatids, and the analogous human enzyme, glutathione reductase, was also inhibited. These experiments were performed *in vitro* and until recently it had not been shown whether trivalent antimonials have an effect in these enzymes *in vivo*. However, it has recently been shown that Sb(III) effects the redox metabolism of *L. donovani* promastigotes in two distinct ways: treatment with the drug caused the rapid efflux of GSH and $T(SH)_2$ from the parasites, together with the intracellular accumulation of oxidised GSH and $T(SH)_2$ (Wyllie *et al.*, 2004). The latter effect is thought to be due to continuing oxidative metabolism in the parasite coupled with inhibition of trypanothione reductase *in vivo*, which, due to the lack of glutathione reductase in parasites, effectively reduces both thiols. The observation that Sb(V) induces similar effects in amastigotes but not promastigotes adds further credence to the hypothesis that toxicity of Sb(V) is dependant upon amastigote-specific reduction of the drug to Sb(III). Interestingly, $T(SH)_2$ levels have been found to be elevated in As(III)-resistant *L. tarentolae* promastigotes (Mukhopadhyay *et al.*, 1996), although this is thought to contribute to increased removal of metalloids from the parasites and will be discussed in section 1.4.5.

1.4.5 Resistance mechanisms and exclusion of antimonials from *Leishmania*

Sb(III) and As(III) can form adducts with GSH and $T(SH)_2$ (Dey *et al.*, 1996; Yan *et al.*, 2003a) and it is these forms which are thought to be extruded from cells (Dey *et al.*, 1996; Legare *et al.*, 2001). How these complexes are formed has not been completely elucidated: they can occur spontaneously *in vitro* (Dey *et al.*, 1996; Yan *et al.*, 2003a) although it has also been proposed that glutathione S-transferases (GSTs) may mediate their formation (Mukhopadhyay *et al.*, 1996) and GST levels were found to be increased in As(III) resistant cells (Lo *et al.*, 1992). Moreover, thiol levels, particularly $T(SH)_2$, were also elevated in As(III)-resistant *L. tarentolae* promastigotes although the increase of $T(SH)_2$ alone is not sufficient to mediate resistance. Although formation and transport of the thiol complexes has not been directly demonstrated for pentavalent metalloids, it is hypothesised that this occurs upon reduction of the Sb(V) (Mukhopadhyay *et al.*, 1996). When incubated with GSH, Sb(V) inhibited the transport of the trivalent metalloid-thiol adducts suggesting that this is indeed the case (Dey *et al.*, 1996). Interestingly, expression of the thiol-synthesis genes encoding gamma-glutamylcysteine synthetase and ornithine decarboxylase were found to be elevated in Sb(V) resistant field isolates (Decuypere *et al.*, 2005). These proteins were also more abundant than in As(III) resistant *Leishmania* (Grondin *et al.*, 1997; Haimeur *et al.*, 1999), resulting in increased levels of $T(SH)_2$ (Haimeur *et al.*, 1999).

How the parasite actually eliminates the metalloid-thiol complexes has not been fully elucidated and, indeed, whether they are actually pumped out of the cell or sequestered in a parasite organelle is also uncertain. Pgpa, an ATP-dependant ABC transporter, has been shown to transport As(III) complexed with GSH (Legare *et al.*, 2001). The location of Pgpa within the parasite was determined by expressing a GFP-fusion protein in *L. tarentolae* and rather than being plasma membrane bound, the protein was situated in an intracellular membrane. This lead to the hypothesis that Pgpa transports trivalent metalloids bound to thiols into parasite vesicles (Legare *et al.*, 2001). The fact that levels of Pgpa have been shown to be amplified in several different studies of antimonial-resistant *Leishmania* adds credence to this theory: MGA-resistant field isolates of *L. viannia* were found to over-express Pgpa on an extrachromosomal amplicon (Anacleto *et al.*, 2003) and Pgpa expression was increased in SSG-resistant *L. donovani* field isolates (Singh, 2006) and in *L. infantum* amastigotes (El Fadili *et al.*, 2005). Moreover, in this latter study, *L. panamensis* amastigotes engineered to over-express Pgpa exhibited increased resistance to Sb(V) when incubated in amastigotes. However, in separate studies Pgpa has been show to not be the transporter responsible for transporting trivalent As(III)-

thiol adducts from *Leishmania*: amplification of Pgpa did not correlate with increased transport of the complexes (Dey *et al.,* 1994) and membranes purified from a Pgpa knockout *L. tarentolae* strain were able to transport the complexes at the same level as those prepared from wild type cells (Dey *et al.,* 1996).

Other transporters that may have a role in eliminating metalloids from *Leishmania* have been identified in other organisms. Acr3p, one of a cluster of three genes (one of which is the arsenate reductase Acr2p) found in yeast was found to be a membrane transporter involved in arsenite transport (Wysocki *et al.,* 1997). Over-expression of Acr3p resulted in *S. cerevisiae* exhibiting increased tolerance to, and decreased accumulation of, arsenite. Accordingly, disruption of the *acr3* gene resulted in increased sensitivity to arsenite and an increased cellular concentration of the trivalent metal was observed (Wysocki *et al.,* 1997; Ghosh *et al.,* 1999). In addition to this, expression of Acr3p is induced by arsenite (Wysocki *et al.,* 2004). Another method of trivalent metalloid extrusion from cells was identified in *E. coli* and is via an ATP-dependant efflux pump called ArsAB. The ArsA subunit possesses the ATPase activity of the pump and is activated by arsenite or antimonite (Hsu and Rosen, 1989). Antimonite binding triggers a conformational change in ArsA and increases the activity of the ATPase 100 fold (Walmsley *et al.,* 2001). The ArsB subunit is a transmembrane protein that is situated in the inner membrane of *E. coli* and translocates the substrate (Wu *et al.,* 1992). No ArsB homologues are known in eukaryotes but *S. cerevisiae* does encode an ArsA homologue termed Yd1100p. The growth of mutants lacking this gene is impeded in the presence of arsenite and antimonite so it has been hypothesised that it may have a role in metalloid export (Tamas and Wysocki, 2001).

Aims of this study

In this chapter it has been described that although pentavalent antimonial compounds have been used for decades to treat leishmaniasis, many aspects of how the drugs function are unknown. The principal aim of this investigation was to identify and characterise a *Leishmania* enzyme able to reduce pentavalent antimonial compounds and therefore mediate toxicity of the drug. Whether such a protein had an *in vivo* role in antimonial susceptibility, and therefore drug-resistance, would be assessed. The following list of objectives was set out in order for the aims to be met:

- Use the sequences of known arsenate reductases to search the *L. major* genome for similar genes which may encode an antimonial reductase
- Elucidate the complete open reading frames of any promising sequences
- Clone potential antimonial reductase genes found and produce soluble, active, recombinant protein
- Characterise the activities of the recombinant protein and raise antibodies to it
- Investigate the *in vivo* spatial and temporal expression profile of any protein studied using the specific antibodies
- Create *Leishmania* parasites that over-express the protein or lack it completely due to the corresponding gene being amplified or knocked out, respectively
- Use the transgenic parasites to ascertain whether the protein identified has a role in susceptibility to pentavalent antimonial drugs and to study the physiological role of the protein in the cell

By using these approaches it was hoped that this investigation would yield a greater understanding of the *Leishmania* proteins involved in antimonial metabolism and their role in drug sensitivity. The findings are presented in chapters 3-5; chapter 6 contains an account of a related yet separate study, the aims of which are described in the introduction to the chapter.

2 Materials and Methods

2.1 Parasites

2.1.1 *Leishmania* culture

L. major and *L. mexicana* promastigote cultures were grown and maintained in an identical manner. Parasites were cultured in HOMEM medium (GibcoBRL, Paisley) with 10% (v/v) heat inactivated foetal calf serum (HIFCS) at 25°C, with air as the gas phase and without shaking. *L. infantum* promastigotes were grown in the same way, except 20% (v/v) HIFCS was used and cultures were incubated at 27 °C. Cells were routinely sub-passaged after the parasites had existed in stationary growth phase (~2 x 10^7 cells/ml) for several days, so approximately every two weeks. New cultures were inoculated at 10^5-10^6 cells/ml depending on the intended purpose for the parasites.

L. mexicana amastigotes were also grown axenically according to the method described previously (Bates *et al.*, 1992). Parasites extracted from lesions were deposited in Schneider's Drosophila Medium (GibcoBRL, Paisley) (pH altered to 5.5 with HCl) with 20% (v/v) HIFCS and 30 μg/ml gentamicin and incubated at 32 °C, again with air as the gas phase and without shaking. Cultures were routinely sub-passaged approximately once a week.

The densities of all cultures were determined by first diluting to approximately 1-2 x 10^6 parasites/ml, then counting using an improved Neubauer haemocytometer (Weber Scientific, Hamilton, NJ, US).

2.1.2 Preparation of *Leishmania* from mice

When fresh cultures that had not been previously sub-passaged were required, *L. major* parasites were extracted from mouse footpads several weeks after parasite innoculation. Parasites were retrieved by first dissecting and then scraping the inside of the footpad, after which the collected material was deposited in a flask containing HOMEM medium with 10% (v/v) HIFCS and gentamicin (Sigma, Poole, UK) at 30 μg/ml. Fresh cultures were then incubated at 25°C as above.

Leishmania major amastigotes purified directly from mouse lesions (Hart *et al.*, 1981) were used to determine the expression of proteins in this particular stage of the life cycle.

Parasites were grown in Balb/c mice and purified by S. Baillie and D. Laughland of the University of Glasgow.

2.1.3 Isolation of metacyclic forms of *Leishmania major*

L. major metacyclic promastigotes were isolated from stationary phase cultures by exploiting the fact that procyclic but not metacyclic parasites are agglutinated in the presence of peanut agglutinin (da Silva and Sacks, 1987). Cultures of known densities were pelleted by centrifugation at 1300 *g* for 10 minutes at 4 °C, washed in PBS and centrifuged again. Pellets were resuspended in PBS at a density of 1 x 10^8 cells/ml and peanut agglutinin (Vector Laboratories, Burlingame, CA, US) was added to a final concentration of 100 µg/ml. After incubation for 30-60 minutes at room temperature the metacyclic-containing supernatant was removed and the parasite density was ascertained.

2.1.4 *Leishmania* harvest and lysis

Parasites were harvested by centrifugation at 1300 *g* for 10 minutes, washed twice in PBS and the resultant pellets were stored at –80 °C until required. *Leishmania* pellets were lysed by resuspending at approximately 1 x 10^9 cells/ml in 1 x GST Bind/Wash Buffer (Novagen, Nottingham, UK) comprised of 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl and 2.7 mM KCl pH 7.3; with the addition of a cocktail of protease inhibitors and chelators: 2 mM ethylenediamine tetraacetic acid (EDTA), 10 µM (2*S*,3*S*)-3-(*N*-{(*S*)-1-[*N*-(4-guanidinobutyl)carbamoyl]3-methylbutyl}carbamoyl)oxirane-2-carboxylic acid (E-64), 2 mM 1,10-phenanthroline, 4 µM Pepstatin A and 1 mM phenylmethylsulfonyl fluoride (PMSF). The samples were kept on ice and immediately lysed by sonication at 20 µm on a cycle of 6 x 10 seconds on, 50 seconds off, using a Soniprep 150 MSE. Successful lysis was confirmed by analysing the samples microscopically and the samples were centrifuged at 1300 *g* for 20 minutes at 4 °C. The soluble fraction (supernatant) and the insoluble fraction (pellet, resuspended in sample buffer) were stored separately at –20 °C until required.

2.1.5 *Leishmania major* cryo-preservation

In order to preserve *L. major* parasite lines for long periods of time – many years in some cases – it was necessary to make stabilates of the parasites. *L. major* cultures in the stationary phase of growth were mixed in a cryotube vial (Nunc, Roskilde, Denmark) with a 1:1 mixture of glycerol and HIFCS in a proportion of 30% mixture and 70% parasites.

The vials were deposited in an isopropyl alcohol bath at room temperature, which was then placed at –70 °C for at least 18 hours before transferring the vials to liquid nitrogen storage (-196°C). The isopropyl bath causes a gradual, uniform temperature decrease to occur, which improves parasite survival.

2.1.6 Bioassays for leishmanicidal activities

In order to determine *L. major* parasite viability following treatment with various compounds, and to calculate the resultant IC_{50} values of these compounds, two different assays were employed.

The acid phosphatase assay relies on *Leishmania* acid phosphatases converting the substrate *p*-nitrophenyl phosphate to *p*-nitrophenol, which is yellow in colour and can be measured using a spectrophotometer at 405 nm (Bodley *et al.,* 1995). The level of acid phosphatase activity is directly proportional to the amount of parasites present in the sample; therefore the assay can be used to analyse the efficacy of compounds on killing *Leishmania* or inhibiting parasite growth. *L. major* parasites were prepared with serial dilutions of the desired compounds in 96-well microtitre plates at a final density of 10^6 parasites/ml, with a volume of 200 µl/well. After incubation at 25 °C for 5-6 days, 20 µl of *p*-nitrophenyl phosphate made up at a concentration of 40 mg/ml in 1 M sodium acetate pH 5.5 with 1% Triton-X100, was added to each well and mixed by aspiration. The microtitre plates were subsequently incubated at 37 °C for 1-2 hours, depending on the progress of the assay as determined by appearance of the yellow *p*-nitrophenol. The absorbance of each sample at 405 nm as determined by reading the plates using a Titertek Multiskan MCC/340 spectrophotometer was used to calculate IC_{50} values using the Grafit (Erithacus, Staines, UK) IC_{50} programme.

The Alamar Blue assay was also employed; this assay has the advantage of not interfering with the viability of the parasites, is very simple and is also easier to judge visually as the colour change involved is more pronounced than that seen in the acid phosphatase assay. Metabolically active *Leishmania* parasites can reduce alamar blue, of which the active ingredient is resazurin (O'Brien *et al.,* 2000), to pink resofurin and the extent of reduction achieved is proportional to the number of parasites present (Mikus and Steverding 2000). Microtitre plates were seeded with *Leishmania* and the desired compounds as for the acid phosphatase assay described above. After incubation at 25 °C for 48 hours, 20 µl of alamar blue (Serotec, Kidlington, UK) or 5 mg/ml resazurin (Sigma, Poole, UK) was added to each well and mixed by aspiration. The plates were subsequently incubated for a further

24-72 hours depending on the progress of the assay as determined by the appearance of the pink resofurin. The absorbencies of the samples at 550 nm, using a reference wavelength of 630 nm, were determined by reading the plates using a spectrophotometer and the IC_{50}s were calculated as above.

2.1.7 *L. major* infectivity

Infectivity of *L. major* lines could be assessed by analysing how well they infected mice. One footpad of each mouse, in groups of up to six animals, was inoculated with either 5 x 10^5 *L. major* promastigotes in stationary phase, or 10^5 *L. major* purified metacyclics, resuspended in 20 µl PBS. The thickness of each infected footpad, which was taken as a direct correlation of parasite burden, was measured on a weekly basis with the help of M. Dixon of the University of Glasgow.

Macrophages were also used to investigate the infectivity of *L. major*. The cells were extracted from the peritoneal cavity of CD-1 mice by S. Baillie of the University of Glasgow and resuspended at a concentration of 5 x 10^5 cells/ml in RPMI medium with 10% (v/v) HIFCS and 30 µg/ml gentamicin. 200 µl of the cell-suspension was dispensed into each well of a chamber slide (VWR, Lutterworth) and incubated at 37 °C in 5% CO_2, 95% air for 24-48 hours. Thereafter, stationary-phase *L. major* diluted in RPMI with 10% (v/v) HIFCS were added to the macrophages at a ratio of 2:1 or 3:1 and incubated as before. After 24 hours the parasite-containing media was removed, the cells were washed several times with media and 200 µl of fresh RPMI with 10% (v/v) HIFCS was added to each chamber. If drugs were being assessed for their antileishmanial ability, they were added to the media at this stage. After incubation for 2-3 days as before, the cells were washed and the media was replaced; the slides were incubated for a total of 5 days. The media was subsequently removed and the cells were fixed by flooding with methanol before being stained with 10% Giemsa stain (Sigma, Poole, UK). The proportion of infected cells, and/or the average number of *L. major* amastigotes infecting each cell, was determined microscopically. At least 150 cells from each chamber were analysed.

2.2 Molecular biology techniques

2.2.1 Isolation of genomic DNA from *Leishmania*

A pellet of 1-2 x 10^8 *Leishmania* promastigotes was resuspended in 400 µl of TELT buffer (50 mM Tris-HCl pH 8.5, 62.5 mM EDTA, 2.5 M LiCl, 4% (v/v) Triton X-100) (Medina-

Acosta and Cross 1993). Trypanosomatid DNA is soluble in this solution and this property is exploited to separate the DNA from unwanted proteins. After five minutes of incubation at room temperature, 400 µl of 1:1 phenol-chloroform (v/v) mixture was added, to give a 1:1 ratio of parasites in TELT buffer to phenol-chloroform, and the sample was mixed by inversion. The sample was then centrifuged at 13000 *g* for five minutes before the aqueous phase was removed and transferred to another microfuge tube. This was then repeated with phenol-chloroform being added to give a 1:1 ratio of DNA-containing aqueous phase to phenol-chloroform. After mixing and centrifugation as before, the aqueous phase was again removed and transferred to a fresh tube and chloroform was added to give a 1:1 ratio of DNA-containing aqueous phase to chloroform. Again, the sample was mixed and centrifuged as before, and the aqueous phase was removed and transferred to a fresh tube for the DNA to be precipitated.

2.2.2 Ethanol precipitation of DNA

Despite commercial kits being used routinely to purify and concentrate DNA, for some procedures ethanol precipitations were also carried out. The volume of the initial sample was determined and 0.1 of the volume of 3 M sodium acetate pH 5.2 was added. The sample was thoroughly mixed and two volumes of ice-cold ethanol were added. Again the samples were well mixed by inversion and incubated at –20°C for at least 15 minutes. The sample was then centrifuged at 13000 *g* for 15 minutes at 4 °C and the supernatant discarded. If the DNA was required for *in vivo* purposes, all subsequent steps were performed under sterile conditions. The DNA pellet was washed twice with 70% (v/v) ethanol; after each wash the sample was centrifuged and the supernatant discarded as before. Finally, after the pellet had been air-dried until no liquid was visible, the DNA was resuspended in the desired volume of TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA) and stored at 4 °C.

2.2.3 Isolation of RNA from *Leishmania*

A pellet of 1-2 x 10^8 promastigotes was resuspended in 1 ml of TR1zol® reagent (GibcoBRL, Paisley) and incubated at room temperature for five minutes. 200 µl of chloroform was added and the suspension was vigorously mixed before being incubated at room temperature for three minutes and then centrifuged at 12000 *g* for 15 minutes at 4 °C. The aqueous phase was then removed and transferred into a fresh microfuge tube into which 500 µl of isopropanol was added before mixing, and incubation at room temperature for 10 minutes. The sample was centrifuged at 12000 *g* for 10 minutes at 4 °C and the

supernatant was discarded leaving the RNA pellet intact. The pellet was washed twice with 1 ml of 70% (v/v) ethanol; after each wash the sample was centrifuged for five minutes at 12000 *g* and the liquid discarded. The pellet was air-dried and, when no liquid remained, resuspended in 50 µl of double distilled water (ddH_2O) which had been treated with 0.01% (v/v) diethylpyrocarbonate (DEPC) in order to remove any trace of RNAse activity.

2.2.4 Rapid amplification of cDNA ends (RACE)

Due to the *L major* genome not having been fully sequenced and, as a result, the database (www.genedb.org) being incomplete when this project commenced, it was necessary to perform 5' RACE on the putative *TDR1* sequence in order to elucidate the entire gene. 5' RACE was also attempted on putative genes designated *LM16* and *LM34* which were also thought to be sequences of interest.

Gene-specific cDNA was synthesised using reagents and according to the protocol from the kit '5' RACE System for Rapid Amplification of cDNA Ends, Version2.0' (Invitrogen, Paisley, UK). Briefly, (using the gene-specific primers OGST1 for *TDR1*, LM16 GSP1 for *LM16* and LM34 GSP1 for *LM34*) cDNA was transcribed from total *L. major* RNA with SUPERscript II reverse transcriptase. After RNAse treatment to eliminate the RNA, the cDNA was purified using a Glassmax DNA Isolation Spin Cartridge. In *Leishmania*, almost all mRNAs have an identical 18 nucleotides known as the splice leader sequence spliced onto the 5' end. Polymerase Chain Reaction (PCR) was performed to amplify the 5' end of the putative genes using a primer complementary to the splice leader sequence (SL primer) and second gene-specific primers (OGST2 for *TDR1*, LM16 GSP2 for *LM16* and LM34 GSP2 for *LM34*). For *TDR1*, the product of this reaction was used as a template in a second, semi-nested PCR using the SL primer and a third gene-specific primer, OGST3.

For the putative gene *LM34* there was no STOP codon in the partial sequence so 3' RACE was performed to elucidate the C-terminal of the gene. Total cDNA was synthesised from RNA, and the 3' RACE procedure was executed using the kit '3' RACE system for rapid amplification of cDNA ends' (Invitrogen, Paisley, UK), according to the manufacturer's instructions. Briefly, *L. major* RNA was incubated with AP primer (which has an oligo–dt component) and total cDNA was synthesised using SUPERscript II reverse transcriptase before treatment with RNAse to eliminate RNA. To amplify the 3' end of the gene, 3' RACE was performed on the cDNA using the supplied AUAP primer and the gene-specific primer LM34 3' GSP1 primer.

2.2.5 Polymerase chain reaction (PCR)

PCR was frequently used to amplify regions of *Leishmania* DNA. All oligonucleotides (primers) were synthesised by MWG biotech (Ebersberg, Germany) and were all manually individually designed. All primers used, their sequences and predicted melting temperatures, are displayed in table 2.1. Different annealing temperatures, numbers of cycles and elongation times were utilised depending on the PCR being performed; often these variables were initially optimised. A PTC-200 DNA Engine Thermal Cycler (MJ Research (BioRad), San Francisco, CA) was used to perform all reactions. Both the Taq (Promega, Southampton, UK) and the Expand High Fidelity (Roche, Lewes, UK) polymerase systems were used according to the manufacturers' instructions.

RACE primers	**SEQUENCE 5'-3'**	**Tm (°C)**
OGST1	AACAATCAGCTGCGACTCGTG	55.3
OGST2	TACAGCCTCGCCCCTCG	54.3
OGST3	AATAGCGCAGGCACCGTATC	54.4
LM16 GSP1	CCGTCCAAACCACTCC	45.4
LM16 GSP2	GCGCAAACTGCCGCACAAACG	65.1
LM34 GSP1	GCGCTCGATCAGCG	46
LM34 GSP2	GCGCCAGCATGTCATGCAGC	62.1
LM34 3' GSP1	CCCTGCCGGTTCTCTACTGGTTTCG	64.5
LM34 3' GSP2	GAGCTGCGCGAAATCAACC	55.7
AUAP PRIMER	GGCCACGCGTCGACTAGTAC	54.7
AP PRIMER	GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTT	68.7
SL PRIMER	TAACGCTATATAAGTATCAGTTTC	65
TDR1 primers		
JOFLOGST1	TAGCGGCCGCTTACCCGCCCTGGGCCCTCCGTTG	>75
JOFLOGST2	GACATATGGCCGCGCGCGCGCTAAAGCTGTACG	>75
JOFLOGST3	TTGCGGCCGCTTACATTGGCGGCCTCTCCGGAAC	>75
New TDR1 o/e P1	GACCCGGGATGGCCGCGCGCGCGCTAAAGC	>75
New TDR1 o/e P2	CTGGATCCTTACCCGCCCTGGGCCCTCC	74.7
TDR13'FLANKP1	CGCCCGGGAGGCTCACCGAGTGGGTCG	>75
TDR13'FLANKP2	CGAGATCTCTTCACACGGGAGAGCACAGCCG	73.3
TDR15'FLANKP1	GCAAGCTTCAGGAACTCGCTGCGCAGTGATCC	74.9
LmGLO primers		
TDR15'FLANKP2	GCGTCGACCGCAGCGGGCGCACCTCTCTAACG	>75
L-gulo3'flankP1	GACCCGGGGGTACCTAAGATTTTGTGC	65
L-gulo3'flankP2	GCAGATCTCCTCCCCCTCCTTGAGGTG	67.4
L-gulo5'flankP1	GCAAGCTTGTGGGGTGCGGAGTTGAG	68.9
L-gulo5'flankP2	GAGTCGACGGTCAAGGTACAATGCAC	61.7
New L-gulo o/e P1	GACCCGGGATGTCTGCTCATTCTGCGGCCCGTC	>75
New L-gulo o/e P2	CTGGATCCTTACGGCGTGCACGCGGTGCTG	>75

Table 2.1: Compendium of primers used, their sequences and Tm (melting temperatures). A, adenine; T, thymine; C, cytosine; G, guanine.

2.2.6 DNA gel electrophoresis

DNA fragments were resolved by electrophoresis on 0.5-1.5% (w/v) agarose gels containing 0.5 μg/ml of ethidium bromide in 0.5 x TBE (45 mM Tris-borate, 1 mM EDTA). The percentage of agarose was dependent on the expected size of the DNA being analysed, with higher percentages being used for smaller fragments. Gels were run in 0.5 x TBE buffer. The 1 kb Plus DNA ladder (Invitrogen, Paisley, UK) was used and a transillumnator (UVP Laboratory Products, Cambridge, UK) was used to visualise the DNA.

2.2.7 DNA quantification

When it was necessary to calculate the concentration of DNA in a sample, serial dilutions were prepared which were then subjected to DNA gel electrophoresis alongside 0.6 μg of 1 kb Plus DNA ladder. As 8% of the mass is contained in the 1650 base pair (bp) band, approximately 48 ng of DNA are present in this band. The intensities of the bands containing the serial dilutions of the original sample were compared to the intensity of the 1650 bp band and the approximate amount of DNA present, and hence the concentration in the sample, was determined.

2.2.8 Cloning of PCR products

PCR products were generated using Taq and Expand High Fidelity polymerases which both add an A (adenine) nucleotide to the 3' end of the DNA fragment being synthesised (known as an A-overhang). The pGEM-T Easy vector (Promega, Southampton, UK) can be used to clone such fragments as it contains a T- (thymine) overhang at the 3' end. PCR products were either isolated using a QIAquick gel extraction kit (Qiagen, Crawley, UK) after DNA gel electrophoresis, or purified using a QIAquick PCR purification kit (Qiagen); in both cases according to the manufacturer's instructions. Approximately 150 ng of the purified product was ligated into 50 ng of the pGEM-T Easy vector using the supplied T4 ligase, usually incubating overnight at 16 °C. Subsequently about 4 μl of the ligation was used to transform 50 μl DH5α competent cells (Invitrogen, Paisley, UK).

2.2.9 Subcloning of DNA fragments

Approximately 1-2 μg of donor and recipient plasmid DNA was digested with up to 50 U of the appropriate restriction enzymes in 100 μl. If digesting with more than one enzyme the most compatible buffer was used, as ascertained by the Promega restriction enzymes

resource (www.promega.com/guides/re_guide/RESearch.asp?search=buffer). If there was no compatible buffer, after the first digest the DNA was recovered using a PCR purification kit (Qiagen) and the second digest was performed on the purified sample. The digested DNA fragments required were recovered by band extraction using a QIAquick gel extraction kit (Qiagen) after separation on an agarose gel. Ligations of the donor and recipient DNA were performed as described, before transformation into DH5α competent cells (Invitrogen).

2.2.10 Restriction digests

All restriction digests were performed using enzymes from Promega, Southampton, UK, according to the manufacturer's instructions. For diagnostic digests approximately 250 ng of DNA was digested with 10-20 U of one or two enzymes in a total reaction volume of 20 µl, usually for two hours at the temperature recommended by the manufacturer. Restriction digests of DNA intended for cloning were performed in larger volumes with additional enzyme and DNA (see section 2.2.9). These digests were performed overnight at the appropriate temperature. Typically the buffer supplied with the enzyme by the manufacturer was used; however, for digests with more than one enzyme a compatible buffer was sought (see section 2.2.9).

2.2.11 Ligations

Ligations of PCR products into the pGEM-T Easy vector (Promega) were performed using the supplied T4 ligase. For all other ligations the T4 DNA ligase from Roche Diagnostics, Lewes, UK, was used. Ligations were performed at a 3:1 (w/w) ratio of donor (insert) to recipient (plasmid) DNA using 2 U of ligase in a 20 µl volume. The reactions were usually incubated overnight at 16 °C although occasionally ligations were performed at room temperature for two hours. For a summary of vectors used, see table 2.2.

Vector	Origin	Information and Application
pGEM-T Easy	Promega, Southampton, UK	A commercially available vector that contains T-nucleotide overhangs for cloning PCR products.
pET28a(+)	Novagen, Nottingham, UK	A commercially available *E. coli* expression vector used to express recombinant TDR1.
pGL345	Derivative of pXG vector (Ha *et al.*, 1996)	Contains cassette that can be used to create gene knockouts in *Leishmania* by homologous recombination. Hygromycin resistance marker.
pGL842	Derivative of pXG vector	As pGL345 but with blasticidin resistance marker.
pGL1033	Derivative of pXG vector	As pGL345 but with phleomycin resistance marker.
pGL102	Derivative of pXG vector	A *Leishmania* expression vector containing a neomycin resistance marker.

Table 2.2: Summary of vectors used, their origins and applications

2.2.12 Plasmid DNA extraction

Plasmid DNA was extracted from *E. coli* using Qiaprep Spin Miniprep and midiprep (when several micrograms of DNA were required) kits (Qiagen) according to the manufacturer's instructions. The volumes of the bacterial cultures used were 3 ml and 35 ml, respectively. The DNA obtained was stored at 4 °C for periods of up to several days, or –20 °C for longer-term storage.

2.2.13 DNA sequencing

DNA sequencing reactions were carried out both by the Molecular Biology Support Unit (University of Glasgow), and by GRI Genomics (Braintree, UK). Sequences were analysed using the Vector NTI programme, version 6.0 (Informax, Bethedsa, MA, US).

2.2.14 Competent cells

For routine cloning, ready-made Library Efficiency DH5α competent cells (Invitrogen) were used according to the manufacturer's instructions.

For recombinant protein expression in *E. coli*, BL21(DE3) cells were made competent manually, being freshly prepared prior to use. 5 ml LB broth was inoculated with a single colony picked from a drug-free LB agar plate and was grown overnight at 37 °C. Thereafter 500 µl of this culture was used to inoculate 50 ml LB broth which was then

grown again at 37 °C. The optical density (OD) of the culture at 600 nm was monitored until the value was between 0.6 and 0.8; the culture was then cooled on ice for 10 minutes before being centrifuged at 2500 *g* for a further 10 minutes. The cells were resuspended in 25 ml of ice-cold 0.1 M $CaCl_2$, chilled on ice for an additional 20 minutes and centrifuged at 2500 *g* for 10 minutes. The cells were finally resuspended in 2.5 ml of ice-cold $CaCl_2$ and stored on ice (or overnight at 4 °C) until required.

2.2.15 Transformation of competent cells

The antibiotics used for selection purposes when transforming bacterial cells were ampicillin, used at 100 µg/ml and kanamycin, used at 50 µg/ml. Both drugs were made up in water at 1000x the working concentration, and stored at –20 °C. Transformations were spread onto LB agar (Sigma, Poole, UK) plates, which were made at 3.5% (w/v). Bacteria were grown in LB broth (1% (w/v) NaCl, 1% (w/v) Tryptone (Sigma), 0.5% (w/v) yeast extract (Sigma).

DH5α cells (Invitrogen) were transformed according to the manufacturer's instructions, with 50 µl cells being used per transformation, 300 µl of the supplied SOC media being added after heat-shock and 140 µl of each reaction being spread on a LB agar plate containing the appropriate antibiotic. When pGEM-T Easy was the plasmid used for the transformation, a blue/white selection procedure was employed. 40 µg/ml isopropylthiol-β-D galactoside (IPTG) and 40 µg/ml 5-bromo-4-chloro-3-indolyl- isopropylthiol-β-D galactoside (X-gal) were added to the LB agar: resulting white colonies correspond to positive clones containing a DNA insert.

When BL21(DE3) cells were being transformed, just 1 µl of a DNA miniprep which had been diluted 1/10 was added to 100 µl of the competent cells. After being incubated for 30 minutes on ice, the samples were heat-shocked at 42 °C for 90 seconds before being cooled on ice for two minutes. 900 µl of LB broth was added to each transformation and mixed, before being transferred to a universal tube so better aeration would be achieved when the samples were incubated at 37 °C for one hour while shaking at 250 rpm. Finally 100 µl of each was spread on a LB agar plate that contained the appropriate antibiotic drug.

2.2.16 Cryo-preservation of bacterial cultures

In order for cloned genes and constructs to be preserved, glycerol stocks of bacterial cultures carrying plasmids, which contained the DNA of interest, were made. A sterile

solution of 40% (v/v) glycerol, 60% LB broth was made. Bacterial cultures, grown overnight in LB broth containing the necessary antibiotics, were mixed with this solution in a 1:1 ratio (v/v) in 1.5 ml screw-cap tubes (Greiner Bio-one, Stonehouse, UK) and stored at –80 °C for up to three years.

2.2.17 Creation of transgenic *L. major* promastigotes

The method used to transfect and generate *L. major* parasites was broadly the same as the high-voltage protocol developed by Steve Beverley and colleagues (Robinson and Beverley, 2003).

For creating lines overexpressing a gene, the parasites were transfected with at least 2 μg of plasmid DNA that contained the gene of interest. However, to knock out a gene from *Leishmania*, linear DNA is used, comprising the 5' flank of the target gene, the 5' flank of the DHFR gene, a drug resistance gene, the 3' flank of the DHFR gene and the 3' flank of the target gene (see figure 2.1). The gene-specific flanks were cloned into the appropriate vector and the total construct excised from the plasmid by restriction digest. The digested sample was then subjected to gel electrophoresis and the desired DNA construct was purified by gel extraction. At least 5 μg of the purified DNA was used for each transfection. In both cases, the DNA to be used for transfecting the parasites was ethanol precipitated before use and resuspended in sterile TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA).

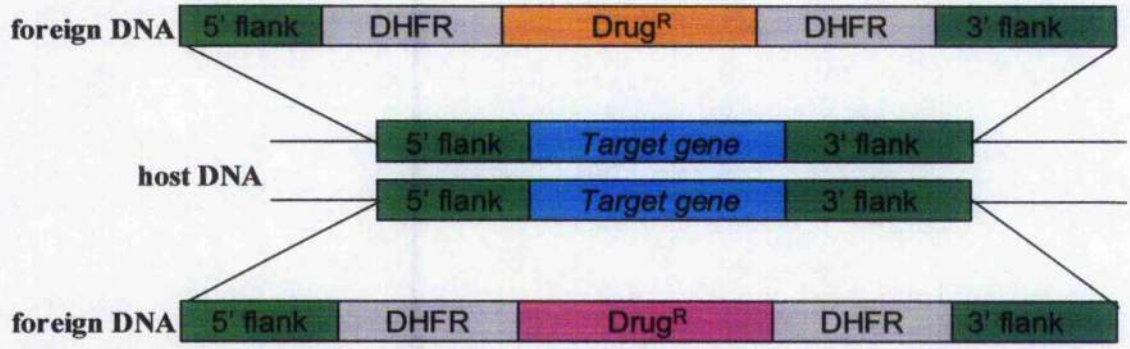


Figure 2.1: Schematic diagram of target locus and constructs introduced when generating a gene knockout in *Leishmania major*. The diagram shows the two copies of the native locus from this diploid parasite and the constructs which replace the genes after two rounds of homologous recombination. The DHFR flanks are included in the constructs as the correct upstream and downstream elements for successful expression of the drug resistance gene.

When the transfections were to be performed, *L. major* promastigotes from the late log/early stationary phase of growth were centrifuged at 1300 *g* for 10 minutes at 4 °C, washed in ice-cold elecroporation buffer (120 mM KCl_2, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4,

25 mM HEPES, 2 mM EDTA, 5 mM $MgCl_2$; pH 7.6) and centrifuged again under the same conditions. The parasites were then resuspended in the chilled electroporation buffer at a concentration of about 2 x 10^8 cells/ml and kept on ice until required. The prepared DNA was deposited into a 4 mm Gene Pulse cuvette (BioRad, Hemel Hempstead, UK) and 0.5 ml of the parasite was added; the sample was briefly aspirated to mix. The cuvette containing the mixture was electroporated twice at 25 μF, 1500 V (3.75 kV/cm) on the BioRad Gene Pulser II apparatus, with a 10 second interval between each pulse. The cells were then removed from the cuvette, transferred into 5 ml of HOMEM medium with 10% (v/v) HIFCS and incubated overnight at 25 °C. The following day the parasites were passaged into the medium containing the appropriate drug(s) (see table 2.3) for selection of successful transformants. 1 ml of the original culture was passaged into 5-10 ml of the fresh, drug-containing medium and the drug(s) were also added to the remainder of the original culture. In all cases, the passaged rather than the original culture contained transformants and was used for subsequent analysis.

For gene knockouts, it was desirable to obtain clones of successfully transformed parasites so a monoclonal culture could be created. This allowed the ensuing parasitic culture to be grown without the selection drugs, and ensured that all parasites within any culture were missing the gene. Clonal populations were obtained by both serial dilution of the original overnight culture from 1/2 to 1/1024 (v/v) in 24-well plates, and by dilution of the successfully transformed passaged culture so that 0.1 parasite was added to each well of a 96-well plate. In both cases, the parasites were added to the plates with the appropriate antibiotic drugs and wells containing growing populations of parasites were accepted as clonal. When serial dilutions of the parasites were made, populations were accepted as clonal when the previous two wells did not contain growing populations.

Drug	Working concentration	Supplier
Blasticidin S Hydrochloride	10-20 μg/ml	Invitrogen, Paisley, UK
Hygromycin B	50-100 μg/ml	Roche, Lewes, UK
Neomycin	50 μg/ml	Calbiochem, Nottingham, UK
Bleomycin	10 μg/ml	Calbiochem, Nottingham, UK

Table 2.3: Antibiotic drugs used for selection of transgenic parasites.

2.2.18 Southern blot analysis

Southern blotting allows detection of specific sequences within genomic DNA and was employed to verify whether the *LmGLO* gene had been successfully knocked out of transgenic *L. major* parasites. Using a combination of Artemis and Vector NTI software, restriction enzymes were chosen that would generate different DNA fragment sizes

depending on whether the intact WT locus was present, or whether homologous recombination had taken place and replaced the gene with DHFR flanking regions and a drug resistance marker. Parasite genomic DNA was extracted and digested to completion using the specific restriction enzymes and DNA fragments were separated on a 1% (w/v) agarose gel in 0.5 x TBE buffer. After electrophoresis for 6 hours at 70 V, the gel was incubated in 125 mM HCl for 20 minutes under gentle agitation, which results in the depurination of the DNA. Subsequently the gel was incubated for 30 minutes in 1.5 M NaCl, 0.5 M NaOH (for denaturation of the DNA) and 30 minutes in 1.5 M NaCl, 1M Tris, pH 7.5 (for neutralisation). These incubations were interspersed by 10 minute washes in ddH_2O, and followed by a 20 minute pre-incubation in 20 x SSC (300 mM tri-sodium citrate, 3 M NaCl, pH 7.0). The DNA was then transferred overnight by capillary action onto Hybond N+ nylon membrane (Amersham) in 20 x SSC, using standard methods (Sambrook *et al.*, 1989). The DNA was fixed onto the membrane by UV cross-linking (Spectrolinker XL-1000 UV linker, Spectronics Corporation, Westbury, NY, US). Until this point, all procedures were performed at room temperature.

The membrane was then pre-incubated at 65 °C for 4 hours in 20 ml Church Gilbert hybridisation solution (340 mM Na_2HPO_4, 158 mM NaH_2PO_4, 240 mM SDS, 1 mM EDTA) supplemented with 0.2 mg/ml salmon sperm DNA (Invitrogen, Paisley, UK) for the final 2 hours of the incubation. 30 ng of nucleic acid probe was prepared from gel-purified restriction fragments using the Prime-It II Random Primer Labelling Kit (Stratagene, La Jolla, CA, US) according to the manufacturer's instructions. With the dATP buffer from the kit, 50 µCi of α32dATP (Perkin Elmer, Beaconsfield, UK) was used to label the A nucleotides of the probe. It was then purified using a Microspin S-200 HR column (Amersham, Chalfont St. Giles, UK) and boiled for 5 minutes before incubation on ice for 2 minutes. The membrane was hybridised by incubation with the labelled probe in 20 ml of Church-Gilbert solution at 65 °C overnight. The membrane was then washed twice under high stringency conditions in 2 x SSC, 0.1% (w/v) SDS, followed by 15 minutes in 1 x SSC, 0.1% SDS and finally twice for 10 minutes in 0.1 x SSC, 0.1% SDS. All washes were performed at 65 °C, using pre-heated solutions. The membrane was then sealed in polythene and exposed to X-ray film for 1-14 days. Hybridisation signals were detected with an automatic film processor (X-Ograph imaging system Compact X4).

2.3 Biochemical methods

2.3.1 Recombinant protein expression in *E. coli*

As mentioned, the bacterial expression plasmid pET28a(+) (Invitrogen) was used to express recombinant proteins; this vector adds an N-terminal His-tag to the expressed polypeptide. The proteins were expressed in the *E. coli* strain BL21(DE3).

The primers FLOGST1 and FLOGST2 were used to amplify the complete *TDR1* gene while the primers FLOGST2 and FLOGST3 were used to amplify the 5' half of the gene. These constructs were cloned into pET28a(+) and the BL21(DE3) cells were transformed with the plasmids as described. Successfully transformed colonies were grown up overnight with kanamycin at 50 μg/ml and 500 μl of each was used to inoculate 50 ml of LB broth containing kanamycin as before. These were shaken at 37 °C and the OD_{600} of the growing cultures was monitored. When the OD_{600} had reached 0.6-0.9, the cultures were cooled on ice for several minutes before IPTG was added to a final concentration of 2 mM. These cultures were grown overnight at 15 °C before being centrifuged at 3000 *g* for 20 minutes at 4 °C. The pellets were resuspended in 5 ml of 20 mM Tris-HCl with 100 mM NaCl and lysed by sonication at 22 μm, on a cycle of 5 x 10 seconds on, 30 seconds off, using a Soniprep 150 MSE. 1 ml of each sample was then centrifuged at 13000 *g* for 20 minutes at 4 °C and the supernatant (soluble fraction) was separated from the pellet (insoluble fraction). The pellet was resuspended in 0.5 ml of 1% SDS and both fractions were analysed by SDS-PAGE for protein expression. In order to achieve optimal expression of the proteins, trials were also conducted with different concentrations of IPTG and growth times.

2.3.2 Recombinant protein purification

Purification of recombinant TDR1 from *E. coli* cells was performed by Alan Scott and Dr. Helen Denton, both University of Glasgow. Expression of TDR1 was induced with 2 mM IPTG and cells were grown overnight and harvested as before, prior to resuspending in buffer A (20 mM Tris-HCl, 500 mM NaCl) containing 5 mM imidazole. The cells were lysed by sonication (a cycle of 10 x 30s at 22 μm) before centrifugation at 13000 *g* for 30 minutes at 4 °C and the soluble supernatant was collected. The soluble fraction was applied onto a 13 ml Ni^{2+}-nitrilotriacetate BioCAD 700E workstation (PE Biosystems, Foster City, CA, US) that had been pre-equilibrated in buffer A containing 5 mM imidazole. The column was washed with 100 ml containing 5 mM imidazole before washing again with 20

ml of buffer A containing 50 mM imidazole. The His-tagged recombinant TDR1 was eluted with 500 mM imidazole in buffer A. The fractions containing the majority of the eluted proteins were pooled and buffer-exchanged into buffer B (25 mM Tris-HCl, pH 7.9) using a PD10 column (Amersham, Chalfont St. Giles, UK) and applied to POROS 20 HQ column (4.6 mm x 100 mm) pre-equilibrated in buffer B. The column was washed with 5 column volumes of buffer B and bound protein was eluted with 10 volumes of buffer B containing a 0-100% gradient of 2 M NaCl. Fractions containing protein were stored at –80 °C.

2.3.3 His-tag cleavage

The pET28a(+) plasmid, used for expressing recombinant TDR1, adds an N-terminal His-tag and thrombin cleavage site to the protein produced. The His-tag can be removed by incubating the purified recombinant protein with biotinylated thrombin, which can in turn be removed by adding streptavidin agarose to the sample and isolating the supernatant after centrifugation. This procedure was carried out using a Thrombin Cleavage Capture Kit (Novagen, Nottingham, UK) according to the manufacturer's instructions. Briefly, 8 U of thrombin was added to 5 mg of TDR1 in 1 x Thrombin Cleavage/Capture Buffer and incubated for 18 hours at room temperature. 16 µl of streptavidin-coated beads were added to the sample, incubated with gentle shaking for 30 minutes at room temperature and centrifuged for 5 minutes at 500 *g*. The supernatant was collected and buffer-exchanged into 25 mM Tris-HCl, pH 7.9 using a PD10 column (Amersham, Chalfont St. Giles, UK). The successful removal of the His-tag was confirmed by a decrease in size of the recombinant protein visible after SDS-PAGE.

2.3.4 SDS-PAGE

Proteins were separated according to their molecular masses under reducing conditions by SDS-PAGE. The protein samples were mixed with 25% volume of 5 x protein sample buffer (60 mM Tris-HCl pH 6.8, 12% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 0.25% (w/v) bromophenol blue) and boiled for three minutes. The samples were resolved by electrophoresis on 8-12% polyacrylamide resolving minigels with 5% polyacrylamide stacking gels using the Mini-Protean II slab system (BioRad, Hemel Hempstead, UK), according to the manufacturer's instructions. The proteins were then either detected by staining or transferred onto membranes for specific protein detection by western blotting.

2.3.5 Detection of proteins on polyacrylamide gels

In order to observe proteins that had been resolved by SDS-PAGE, gels were routinely stained with 0.25% (w/v) Coomassie Brilliant Blue R250 in 10% (v/v) methanol and 12.5% (v/v) isopropanol, usually overnight although occasionally for just 1-2 hours. Gels were destained with 10% (v/v) acetic acid, 10% (v/v) methanol, for several hours, usually with several changes of destain. Destained gels were visualised with a light-box (Hancocks, UK).

When increased detection of proteins was required, silver staining was employed. Gels were initially fixed in 40% (v/v) methanol and 10% (v/v) acetic acid for 30 minutes at room temperature, followed by two incubations for 15 minutes at room temperature in 10% (v/v) ethanol and 5% (v/v) acetic acid. To oxidise the proteins, the gels were incubated in 5% (v/v) glutaraldehyde solution, for five minutes at room temperature. The gels were washed twice in distilled water for five minutes at room temperature before reducing in 0.4% (w/v) $AgNO_3$ for 20 minutes at room temperature. Gels were briefly washed in distilled water and developed in 0.02% (v/v) formaldehyde and 2.5% (w/v) Na_2CO_3. When the proteins became sufficiently visible the development was terminated in 5% (v/v) acetic acid; the gels were also stored in this solution.

2.3.6 Protein concentration determination

Protein concentrations in both cell lysates and purified recombinant protein samples were determined using the BioRad protein assay, based on the Bradford method (Bradford 1976). Bovine serum albumin (Promega, Southampton, UK) was used as the protein standard; graphs and protein concentration calculations were made using Grafit software (Erithacus, Staines, UK).

2.3.7 Confirmation of axenic amastigotes by protease expression profile

Although it is possible to grow axenic amastigote-like forms of *L. mexicana*, it is necessary to confirm their similarity to amastigotes obtained from animals, rather than promastigotes grown in culture. This can be achieved by comparing the protease expression of the different parasite forms profile by gelatin SDS-PAGE (Bates *et al.*, 1992).

SDS-PAGE was performed as described in section 2.3.4, except in addition the gels contained 0.2% (w/v) gelatin. Following electrophoresis, the gels were incubated for one

hour at room temperature in 2.5% (v/v) Triton X-100 and then in 0.1 M sodium acetate pH 5.0 with 10 mM DTT at 37 °C for 1.5 hours. The gels were stained in coomassie blue, as described in section 2.3.5.

2.3.8 Antibody production

Before the complete *TDR1* gene had been identified, the 3' half of the gene was assumed to be the whole coding region and was cloned and expressed. The resulting N-terminal His-tagged, purified recombinant protein was unstable, but was used as an antigen to obtain antiserum from rabbit. This procedure was performed by the Scottish Antibody Production Unit (Carluke, UK), using standard methods. The antibody obtained was subsequently purified (see section 2.3.6).

Herein after it was decided a second anti-TDR1 antibody should be generated, this time using the recombinant protein obtained upon expression of the whole gene. Again, N-terminal His-tagged, purified recombinant protein was used as the antigen, this time to produce antiserum in sheep. This procedure was performed by Diagnostics Scotland (Edinburgh, UK), using standard methods.

For production of an antibody raised against the putative LmGLO protein, the facility used was Biological Services, University of Glasgow. N-terminal His-tagged recombinant protein was produced and purified by Dr. Helen Denton and Dr. Gareth Westrop (both University of Glasgow); 50 µg protein diluted 1:1 (v/v) in Freund's adjuvant (Sigma, Poole, UK) in a final volume of 100 µl was inoculated into a rat. After one month, this was followed by two more similar booster inoculations.

2.3.9 Antibody purification

As described in the previous section two different anti-TDR1 antibodies were raised, both of which were provided as crude serum. The first (rabbit) anti-serum did not recognise TDR1 from *Leishmania* lysates on western blots although it did recognise many other *Leishmania* lysate proteins along with purified recombinant TDR1 protein. These observations suggested that the anti-serum was not specific to TDR1 but that it did contain anti-TDR1 antibodies albeit at a low titre. Therefore, it was decided to purify and concentrate the anti-TDR1 antibodies using recombinant TDR1.

The protocol for purifying the anti-TDR1 antibody was optimised, so here only the optimised procedure involving an acidic column equilibration and final elution are

described. 10 mg of purified recombinant TDR1 in 2 ml 20 mM MOPS, pH 8.0, was incubated with 2 ml of Affi-gel 15 (BioRad, Hemel Hempstead, UK) overnight at 4 °C. The incubation was centrifuged for one minute at 50 *g* and the supernatant (containing unbound protein) was removed before resuspending the gel in 3 ml of 20 mM MOPS, pH 8.0. 150 µl of 1 M ethanolamine, pH 8.0 was added (this blocks any uncoupled sites) and incubated for a further 1 hour at 4 °C. The sample was applied to a PD 10 column (Amersham, Chalfont St. Giles, UK) which was then washed with 10 ml each of: Tris-HCl, pH 7.5; Tris-HCl, pH 7.5 with 500mM NaCl; and glycine, pH 2.5, all at 100 mM. The column was then washed with Tris-HCl, pH 7.5, until the run-through was also pH 7.5, before 5 ml of heat-inactivated antiserum (heated to 65 °C for 20 minutes) was applied to the column; the run-through was re-applied twice to ensure optimal binding. The column was washed with 20 ml of 10 mM Tris-HCl, pH 7.5 with and without 500 mM NaCl. Finally, the bound antibodies were eluted in 6 ml of 100 mM glycine, pH 2.5.

2.3.10 Western blot analysis

Following separation by SDS-PAGE, proteins were transferred to Hybond-C nitrocellulose membrane (Amersham, Chalfont St. Giles, UK) by electroblotting in transfer buffer (20 mM Tris, 15 mM glycine, 20% (v/v) methanol that had been pre-chilled to 4 °C. The transfer was performed using a mini transblot cell (BioRad, Hemel Hempstead, UK) for 45 minutes at 100 V. A Seeblue protein standard (Invitrogen, Paisley, UK) was run alongside the protein samples; this can be visualised without staining. However, the membrane was stained with Ponceau S as this allowed observation of any air bubbles which could be marked, and confirmation of equal protein content between lanes when required. The membrane was blocked at 37 °C for 1 hour in 1 x TBS (20 mM Tris-HCl, pH 7.6, 13.7 mM NaCl) with 0.1% (v/v) Tween-20 and 5% (w/v) dried milk, before incubating overnight at 4 °C with the appropriate primary antibody or antibodies in 1 x TBS with 0.1% (v/v) Tween-20 and 1% (w/v) dried milk. The following day the membrane was washed for 20 minutes four times at room temperature with 1 x TBS with 1% (w/v) dried milk, before application of the secondary antibody in 10 x TBS with 1% (w/v) dried milk for two hours at room temperature. The membrane was further washed for 20 minutes three times in 1 x TBS with 1% (w/v) dried milk before rinsing briefly in 10x TBS and developing the blot. All secondary antibodies were HRP-conjugated so blots were developed manually by applying Supersignal West Pico substrate (Pierce, Cramlington, UK) and using Hyperfilm ECL (Amersham, Chalfont St. Giles, UK) to expose them, according to the manufacturers' instructions. Films were developed and fixed using Kodak GBX developer/replenisher and fixer/replenisher (Sigma, Poole, UK), respectively.

Antibody	Source	Type	Dilution
rabbit anti-TDR1 (1)	Scottish Antibody Production Unit, Carluke, UK)	primary	1/20 (purified)
sheep anti-TDR1 (2)	Diagnostics Scotland, Edinburgh, UK	primary	1/4000
rat anti-L-GULO	Biological services, University of Glasgow, UK	primary	1/1000-1/10000
rabbit anti-Transketolase	Gifted by Dr. Mike Barrett, University of Glasgow, UK	primary	1/2000-1/10000
rabbit anti-Cysteine Synthase	Gifted by Dr. Rod Williams, University of Glasgow, UK	primary	1/5000
anti-sheep IgG HRP conjugated	Santa Cruz Biotechnologies, CA, US)	secondary	1/5000
anti-rat IgG HRP conjugated	Pierce, Cramlington, UK	secondary	1/2000
anti-rabbit IgG HRP conjugated	Pierce, Cramlington, UK	secondary	1/10000

Table 2.4: Antibodies used in Western Blots

2.3.11 Immuno-localisation of cellular protein

In order to analyse the distribution of TDR1 in *L. major*, immuno-fluorescence was performed using the anti-TDR1 antiserum produced in sheep, using an adapted method from one previously described (Field *et al.*, 2004). Parasites were harvested by centrifugation at 1300 *g* for 10 minutes at 4 °C, washed in vPBS (Voorheis' PBS: 137 mM NaCl, 3 mM KCl, 16 mM Na_2HPO_4, 3 mM KH_2PO_4, 46 mM sucrose, 10 mM Glucose; pH7.6 (Nolan *et al.*, 2000)), and resuspended at ~2 x 10^7 cells/ml in vPBS. An equal volume of 6% (w/v) paraformaldehyde in vPBS was added and the mixture was incubated on ice for one hour. Five volumes of PBS was added and the parasites were harvested and washed as above, before resuspending at ~2 x 10^7 cells/ml in PBS. 200 µl of the parasite suspension was applied to a glass slide that had been pre-treated with 1 ml of 0.01 (w/v) poly-L-lysine solution (Sigma, Poole, UK) for five minutes and allowed to dry for > one hour at room temperature. The parasites were incubated for 15 minutes on the slide before the cells were permeabilised in 0.1% (v/v) Triton X-100 in PBS for 10 minutes at room temperature. Slides were washed at room temperature three times for five minutes in PBS, then blocked for one hour with 20% (v/v) HIFCS in PBS. The sheep anti-TDR1 primary antibody was applied at various dilutions in PBS with 20% (v/v) HIFCS for one hour at room temperature before being washed three times in PBS as before. The secondary antibody (Alexa Fluor 488 donkey anti-sheep IgG, Molecular Probes, Paisley, UK) was

applied at room temperature for one hour at a dilution of 1/500 in PBS with 20% (v/v) HIFCS along with 5 μg/ml 4',6-diamidino-2-phenylindole (DAPI) stain. Slides were washed three times in PBS as before, dried and mounted with 1:1 (v/v) glycerol in PBS. Nail varnish was used to seal the edges of coverslips and slides were stored in the dark before analysis using an Axioplan Fluorescence microscope (Zeiss, Welwyn Garden City, UK) and Hamamstsu digital camera with Openlab software (Improvision, University of Warwick, UK).

2.3.12 Isolation of protein using S-hexyl-GSH agarose

It is possible to pull TDR1 and other proteins out of *Leishmania* lysates by exploiting the fact they bind to hexyl-glutathione. *Leishmania* parasites were harvested, resuspended at ~1 x 10^9 cells/ml, lysed and centrifuged as before. The resultant soluble fraction was incubated with 100 μl/ml S-hexyl-glutathione sepharose (Sigma, Poole, UK) for two hours at room temperature with gentle mixing before centrifugation at 2500 *g* for one minute to sediment the sepharose. The sepharose pellet was vortexed and washed three times in 1 ml of 1 x GST Bind/Wash Buffer (composition as before, Novagen, Nottingham, UK), once in 50 μl of 1 x GST Bind/Wash Buffer and once in 50 μl of 50 mM Tris-HCl, pH 8.0; the latter two washes were retained for analysis. Finally, bound proteins were eluted by adding 50 μl of 10 mM S-hexyl-glutathione (Sigma, Poole, UK) in 50 mM Tris-HCl, pH 8.0, vortexing briefly, centrifuging as before and isolating the supernatant.

A similar method was also used to isolate TDR1 from spent culture media. The method was broadly the same as described above except: 9 ml of spent media was filtered and mixed with 1 ml of 10 x GSH Bind/Wash Buffer and used rather than lysate; incubation with 100 μl of S-hexyl-glutathione sepharose was performed overnight at 4 °C and the final washing step was omitted.

2.3.13 BPR assay for measuring trivalent antimonials

The BPR assay can be used to detect trivalent antimonial species (SbIII). This method used was based on a procedure described previously (Frezard *et al.*, 2001) and was employed to analyse whether H_2O_2 reacted with trivalent antimonials. Briefly, 200 μl of analyte solution comprising 20 mM sodium phosphate, pH 6.8, 0.1% (w/v) tartaric acid and 70 μM Bromopyrogallol Red (Sigma, Poole, UK) was added to 20 μl of sample in a microtitre plate. The absorbance was read at 540 nm using a Titertek Multiskan MCC/340 spectrophotometer. Potassium antimonyl tartrate (0-0.5 mM in 20 μl), made up in the same

buffer as the samples, was used to construct a calibration curve for each experiment performed and tests were conducted to confirm that none of the sample components interfered with the assay. Under the conditions used, this assay was shown to be specific for trivalent antimonial compounds and gave no reaction with sodium stibogluconate, GSH, GSSG or H_2O_2. The standard reaction mixture contained, in a volume of 1 ml, 100 mM Tris-HCl, pH 8.0 (unless otherwise stated) and 1 mM SbIII in the form of potassium antimonyl tartrate (Sigma, Poole, UK). Potential inhibitors along with TDR1 were also tested for their effect on the reaction; these were also present in the reaction mixture. The reaction, performed at room temperature, was initiated by the addition of H_2O_2, and 20 µl aliquots were removed at time-points between 2-180 minutes so the Sb(III) content could be calculated using the BPR assay. Potassium antimonyl tartrate (0-0.5 mM in 20 µl), made up in the same buffer as the samples, was used to construct a calibration curve for each experiment performed.

2.4 Statistical analysis

Values were expressed either as mean ± standard deviation (SD) when the number of repetitions was more than two, or mean ± standard error (SE) when the number of repetitions was two. Significance levels were calculated by unpaired t-tests using the t-test function in the Microsoft Excel programme. Differences were considered significant when the p value was <0.05.

2.5 Bioinformatic analyses

For trypanosomatid database mining and subsequent analysis, the website www.genedb.org was used extensively. The website www.ncbi.nlm.nih.gov and particularly the tblastn and tblastx BLAST search facilities were used for additional database mining and analysis. Examination of *Leishmania* DNA loci, generally of gene flanking regions, was performed using the Artemis programme.

The SignalP (version 3.0) programme (http://www.cbs.dtu.dk/services/SignalP/) was used to determine if genes of interest included any target signalling regions. The related targetP programme (version 1.1) was (www.cbs.dtu.dk/sevices/TargetP) was used to predict the subcellular localisation of proteins deemed to possess a signalling tag.

Vector NTI (version 6.0) software was used to organise, edit and analyse DNA and protein sequences as well as to facilitate the design of oligonucleotides. All sequence alignments

and sequence similarity comparisons were made with alignX. Sequenced DNA was viewed and analysed using ContigExpress.

3 Pursuit of *Leishmania* genes and proteins involved in pentavalent antimonial activation

As discussed in chapter one, the toxicity of pentavalent antimonials, the standard first-line treatment against visceral leishmaniasis, is thought to be dependent on their reduction to trivalent forms. The site of the reduction remains controversial; the amastigote itself has been reported to reduce the drug (Ephros *et al.*, 1999) although conflicting accounts regarding the lack of sensitivity that axenically grown amastigotes display to the drugs has lead to speculation that the host macrophage mediates reduction (Sereno *et al.*, 1998). Whether reduction is enzymatically controlled is another contentious issue as pentavalent antimonials can be directly reduced to trivalent forms upon reaction with low molecular weight thiols such as trypanothione and glutathione *in vitro* (Frezard *et al.*, 2001; Ferreira Cdos *et al.*, 2003; Yan *et al.*, 2003b). However, shortly before this work was initiated it was reported that *L. donovani* amastigotes possess antimonial-reducing activity which coincides with sensitivity to sodium stibogluconate (Shaked-Mishan *et al.*, 2001). Drug-resistant parasites no longer had the capacity to reduce the pentavalent compound. Although providing no direct proof that the observed activity was enzymatic, the stage-specific nature of it combined with the apparent ability of the resistant parasites to dispense with drug-reducing activity suggested that that may well be the case.

No reports exist of proteins that specifically reduce or methylate antimonials; for this reason homologues of genes encoding proteins known to metabolise arsenate were pursued. Arsenate reductase (ars) operons can confer resistance to antimonite when over-expressed (Carlin *et al.*, 1995) suggesting that both compounds can be used as substrates by the encoded proteins. The metabolism of arsenic, which is adjacent to and belongs to the same group as antimony in the periodic table, has been much more extensively studied (probably due to its toxic effects often being seen in humans) and more is known about its toxicology. Accordingly, it is often necessary to consider research carried out on arsenical compounds when investigating antimonials (Gebel, 1997). In addition to the reduction of inorganic, pentavalent metalloids, biomethylation has also been considered as a potential route of pentavalent antimonial drug metabolism. As reviewed in chapter one, the pathway is known to occur in a range of organisms and also involves reduction, albeit of methylated intermediates. Like arsenate reduction, biomethylation of metalloids yields more toxic products and may be considered as an activation pathway. As discussed, mammalian enzymes able to mediate biomethylation have recently been elucidated and, in addition, several different classes of arsenate reductases have been identified and characterised in

eukaryotes and prokaryotes. This chapter details an investigation into whether genes encoding similar proteins to these, potentially involved in antimonial activation, are present in the *L. major* genome.

Sequencing of the *Leishmania major* genome has recently been completed (Ivens *et al.*, 2005) but, together with those of other *Leishmania* species, the genomes have been elucidated over several years with more data being made gradually available. The databases – all found at www.genedb.org – have been frequently searched for sequences similar to the known arsenate-metabolising proteins; results of the most recent searches are summarised in this chapter. Moreover, genes and proteins potentially involved in antimonial reduction have been pursued by additional methods. These, along with the identification of one gene in particular, which is interesting because of both its significant homology to the inorganic arsenical and methylated arsenical reductase omega glutathione S-transferase (oGST) and its unusual sequence features, are also described.

3.1 Arsenate reductase homologues in *L. major*

Through frequent reviewing of available literature, several categories of proteins capable of reducing pentavalent arsenicals were identified. Three separate classes of microbial (two in bacteria and one in yeast) arsenate reductases had been previously characterised and during this investigation two mammalian proteins were found to be capable of reducing arsenate: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and purine nucleoside phosphorylase (PNP). In order to determine whether *Leishmania* may possess a protein capable of reducing pentavalent antimonials, the amino acid sequences of these arsenate reductase enzymes were used as enquiry sequences to search the *L. major* predicted protein database. Searches for *L. major* sequences were conducted using the omniblast feature at www.genedb.org and identified proteins were used as enquiry sequences to search known proteins of the original organism using the blastp facility found at http://www.ncbi.nlm.nih.gov/BLAST/. Alignments were performed using the alignX feature of vector NTI. The results of these blast searches and the ensuing analysis of the sequences identified are presented in this section and summarised in table 3.1.

Enquiry sequence: organism, enzyme name, and amino acid length	***Leishmania* homologues, p/n scores and amino acid length**	**% identity, % conservation and observations on alignments**	**Single organism back BLAST results**
E. coli R773 ArsC (1I9DA), 141 amino acids	**LmjF05.0150** 0.94, 441 amino acids	11%, 33%. *L. maj* seq 240 amino acid N-term ext and small C-term ext.	Enq seq not significantly similar.
1. *S. aureus* pI258 ArsC (AAA25638), 131 amino acids 2. *B. subtilis* pI258 ArsC (NP390455), 139 amino acids	**LmjF01.0200** 1. 0.017, 2. $9.0e^{-6}$ 300 amino acids	**1.** 21%, 37%. **2.** 22%, 37%. *L. maj* seq has small N-term and larger C-term ext, plus insertion.	**1.** N/A **2.** Best hit to enq seq.
S. cerevisiae Acr2p (NP015526) 130 amino acids	**LmjF32.2740** 0.011, 229 amino acids	16%, 31%. *L. maj* seq 99 amino acid N-term ext.	Best hit to enq seq.
Human PNP (NP000261) 289 amino acids	**LmjF05.0830** $7.1e^{-8}$, 306 amino acids	21%, 42%. L. maj seq small C-term ext & small N-term deletion.	10^{th} hit to enq seq. Best is methylthioadenosine phosphorylase (MTAP).
Human GAPDH (NP002037) 335 amino acids	**a. LmjF30.2970** $2.3e^{-86}$, 361 amino acids **b. LmjF30.2980** $5.3e^{-87}$, 361 amino acids **c. LmjF36.2350** $5.7e^{-74}$, 215 amino acids **d. LmjF35.4750** $9.3e^{-21}$, 349 amino acids	**a & b.** 51%, 70%. Good similarity. **c.** 68%, 80%. L. maj seq 120 amino acid N-term deletion. **d.** 26%, 45%. Good similarity.	**a & b.** 2^{nd} hit to enq seq. Best is spermatogenic GAPDH. **c.** Best hit to enq seq. **d.** Best hit to enq seq.

Table 3.1: *L. major* sequences similar to arsenate reductases. Bacterial proteins are coloured in purple, yeast and plant Acr2p is shown in grey-blue, and mammalian sequences are in red. Abbreviations used are PNP – purine nucleoside phosphorylase, GAPDH – glyceraldehydes-3-phosphate dehydrogenase, L. maj – *L. major*, seq – sequence, ext – extension, N-term – N-terminal, C-term – C terminal, enq – enquiry.

3.1.1 Microbial Arsenate Reductases

The two unrelated classes of bacterial ArsC proteins are both encoded on extra-chromosomal plasmids, the most extensively investigated of each being the Gram-positive *S. aureus* pI258 ArsC and Gram-negative *E. coli* R773 ArsC. For clarification purposes these proteins will be herein referred to as pI258 ArsC and R773 ArsC. Together with Acr2p, first found in *S. cerevisiae* and for some time the only known eukaryotic arsenate reductase, these proteins comprise the group designated here as microbial arsenate

reductases. It should be noted that recently Acr2p was identified in plants (Duan *et al.*, 2005; Dhankher *et al.*, 2006) and therefore can no-longer be thought of as purely a microbial enzyme. Although all three proteins have similarities with other enzymes, they have no other identified enzymatic capabilities and are classified specifically as arsenate reductases. This may be of importance when considering similar *Leishmania* sequences.

3.1.1.1 R773 ArsC

The *L. major* genome was searched for sequences similar to the 141 amino acid *E. coli* R773 ArsC protein. The most similar (p/n value = 0.94) predicted protein, LmjF05.0150, was annotated as a hypothetical protein. Comprising 441 amino acids, it was predicted to be more than three times the length of R773 ArsC and have a 240 amino acid N-terminal extension as well as a smaller C-terminal extension, when the two sequences were aligned (figure 3.1). The regions of the sequences that did align shared just 11% identity and 33% conservation. When the amino acid sequence of LmjF05.0150 was used as an enquiry sequence in a blastp search of *E. coli* proteins, the original ArsC sequence was not found to be significantly similar. Other than trypanosomatid proteins, LmjF05.0150 is most similar to NOD3, a caterpillar protein involved in T-cell activation. The crystal structure of *E. coli* R773 ArsC has been previously solved (Martin *et al.*, 2001); Arg60, Arg94, and Arg107 are required to bind arsenate and the actual reduction is dependant on Cys12. Of these residues, none were conserved when the sequence was aligned with that of LmjF05.0150 although Arg60 and Arg107 aligned with the similarly basic amino acids Lys300 and Lys347, respectively. The low percentage similarity together with the high p/n value score given for the two proteins being related, imply that LmjF05.0150 is an unlikely R773 ArsC arsenate reductase, an observation supported by the lack of conservation between important residues and the large difference in predicted protein size. Other sequences identified in the search were less similar to R773 ArsC than LmjF05.0150 and unconvincing matches; these have not been detailed here.

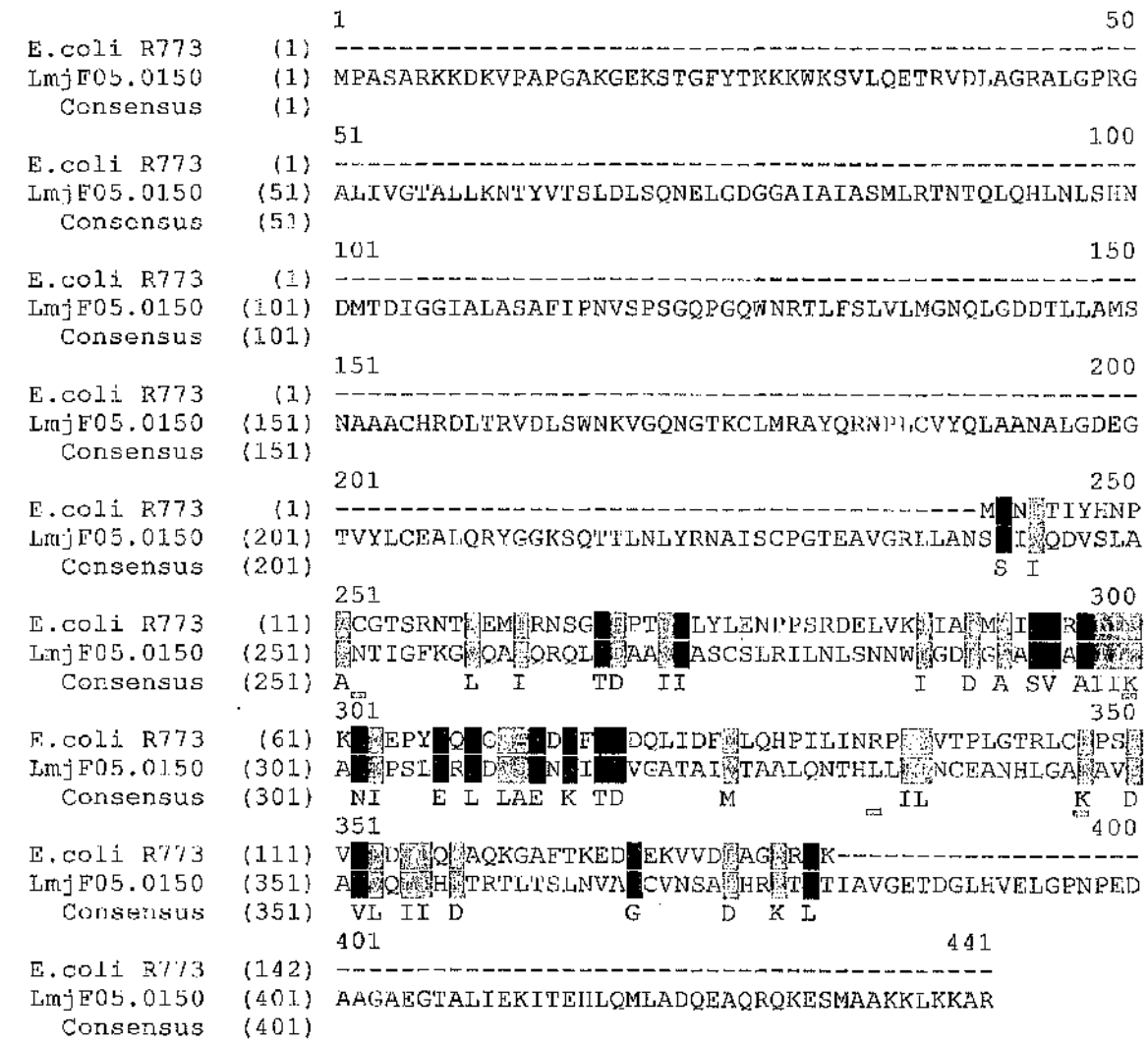


Figure 3.1: Alignment of *E. coli* R773 ArsC with the most similar *L. major* sequence, LmjF05.0510. E. coli R773 – *E. coli* R773 ArsC. The residues known to be essential for arsenate binding and reduction in *E. coli* R773 ArsC are underlined in red; none of these residues are present in the *L. major* sequence. Dashes indicate gaps in the alignment. Identical residues are printed in white on a black background and similar residues are printed in black on a grey background.

3.1.1.2 pI258 ArsC

Using the amino acid sequence of the *S. aureus* pI258 ArsC as the inquiry sequence, the *L. major* genome was searched as above for similar proteins to this second class of ArsC reductases. Again the most alike (p/n = 0.017) protein, LmjF01.0200, was annotated as being a hypothetical protein but was listed as exhibiting sequence similarities to low molecular weight phosphotyrosine protein (LMW PTPs) phosphatases. This was of interest as pI258 ArsC proteins are thought to have evolved from genes encoding these enzymes (Zegers *et al.*, 2001) and the *B. subtilis* pI258 ArsC displays low-level PTP phosphatase activity (Bennett *et al.*, 2001). In order to determine whether LmjF01.0200 was more similar to pI258 ArsC or LMW PTPs, the amino acid sequence was used to search the

protein database at www.ncbi.nlm.nih.gov. Unfortunately the resultant list of alike proteins was divided between predicted arsenate reductases and LMW PTPs. One of these was the *Bacillus subtilis* pI258 ArsC, another experimentally confirmed pI258 ArsC (Sato and Kobayashi, 1998). This amino acid sequence was used to search for similar *L. major* predicted proteins and once again LmjF01.0200 was the most alike. However, the p/n value was given as $9.0e^{-06}$, considerably smaller than when the *S. aureus* protein was used as the inquiry sequence. The sequence of LmjF01.0200 was used as an enquiry sequence in a blastp search of *B. subtilis* proteins and the most similar was the pI258 ArsC. It is not possible to perform a single-organism search on the *S. aureus* genome at present.

Despite the improved p/n value, several factors suggested that the LmjF01.0200 protein may not necessarily be a pI258 ArsC. The *L. major* protein was predicted to contain 300 amino acids, more than twice the number of the *S. aureus* pI258 ArsC (130) or the *B. subtilis* enzyme (139). Accordingly, when the three protein sequences were aligned (figure 3.2), LmjF01.0200 contained a small N-terminal extension and mid-sequence insertion as well as a C-terminal extension of almost 100 amino acids compared to the bacterial protein sequences. Of the areas that align, LmjF01.0200 shares identity with the *S. aureus* and *B. subtilis* pI258 ArsC proteins of 21% and 22% respectively, and conservation of 37% with both proteins. Several residues have been previously identified as being important for activity in both LMW PTP and pI258 ArsC enzymes: all known LMW PTPs contain a CXXXXXRS/T motif (Fauman *et al.*, 1996) while a comparable CTGNSCRS motif is conserved between pI258 ArsC arsenate reductases (Bennett *et al.*, 2001). In addition, Cys82, Cys89 and Asp105 are all required for the *B. subtilis* pI258 arsenate reductase catalytic mechanism (Messens *et al.*, 1999; Bennett *et al.*, 2001). Although Cys82 and Cys89 are conserved in LmjF01.0200 (Cys155 and Cys163, respectively), there is no corresponding aspartic acid. Furthermore, there is no intact CTGNSCRS or even CXXXXXRS/T motif: although the penultimate and final residues are conserved in Arg51 and Thr52, the essential cysteine is absent. Due to the fact LmjF01.0200 lacks the necessary amino acid requirements for both arsenate reductase and PTPase activity, it is unlikely to possess either of these enzymatic capabilities unless a modified or previously unidentified catalytic mechanism is involved.

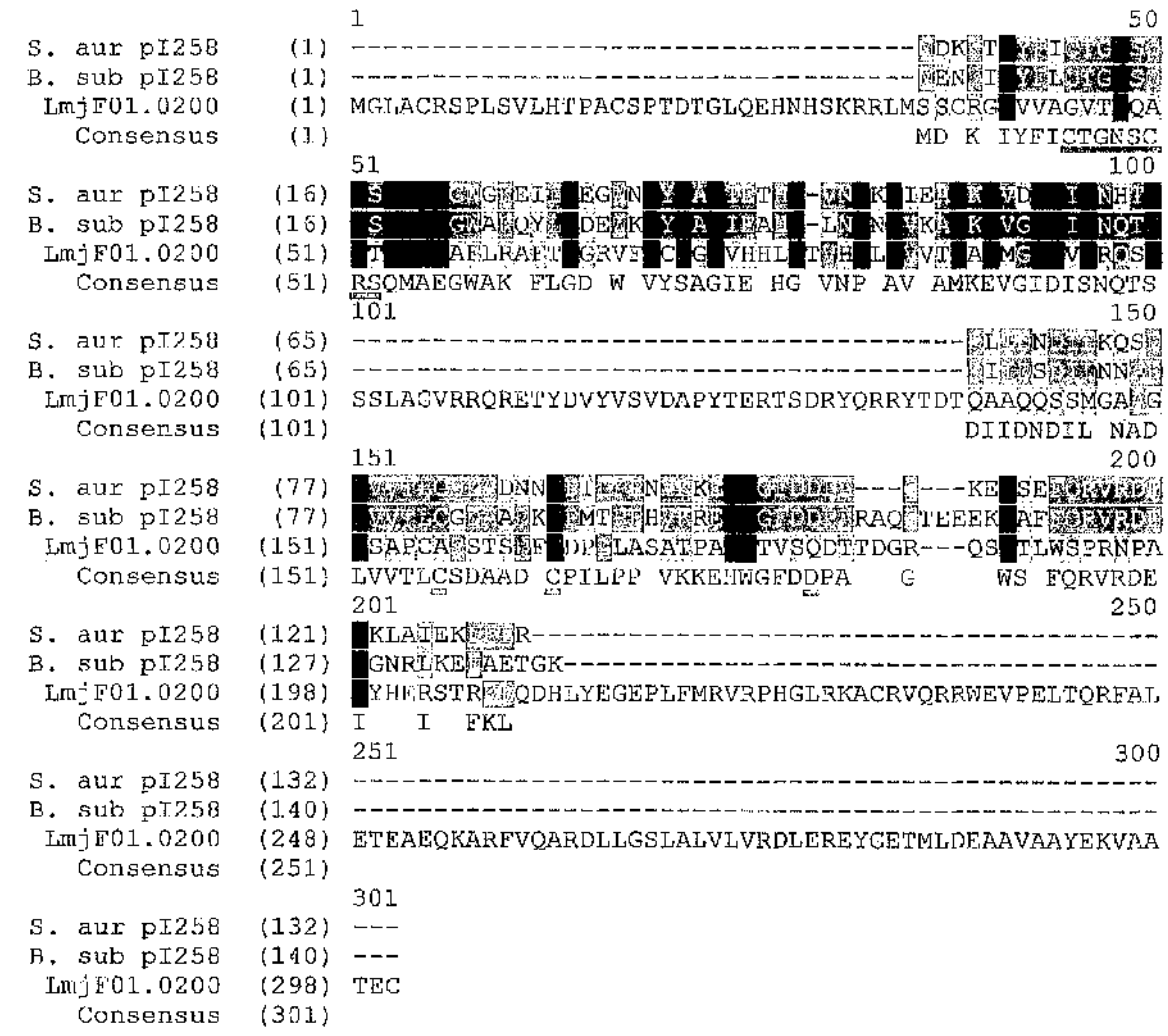


Figure 3.2: Alignment of *S. aureus* and *B. subtilis* ArsC with the most similar *L. major* sequence, LmjF01.0200. S. aur pI258 – *S. aureus* pI258 ArsC, B. sub pI258 – *B. subtilis* pI258 ArsC. The residues known to be essential in the S. aureus and B. subtilis sequences are underlined in blue and the essential motif is underlined in red. Although the *L. major* sequence contains a cysteine residue which aligns with the Cys89 of the known pI258 ArsC sequences and contains another which aligns adjacent to the Cys82 residue, it lacks an aspartic acid residue that aligns with Asp105 and the essential CTGNSCRS motif. Dashes indicate gaps in the alignment. Identical residues are printed in white on a black background, conserved residues are printed in white on a grey background and similar residues are printed in black on a grey background.

3.1.1.3 Acr2p

Acr2p – the third category of microbial arsenate reductases – was first identified in *S. cerevisiae*, and the 130 amino acid sequence of the yeast protein was used here to search the *L major* genome for similar predicted proteins. Once more the most similar sequence was that of a hypothetical protein, LmjF32.2740, and the p/n score given was 0.011. Predicted to comprise 229 amino acids, it was again a considerably larger protein than its microbial counterpart, *S. cerevisiae* Acr2p, and when the two sequences were aligned (figure 3.3) LmjF32.2740 was expected to have a 100 amino acid N-terminal extension.

The sections of the two proteins that did align shared 16% identity and 31% conservation. When LmjF32.2740 was used as an enquiry sequence to search the *S. cerevisiae* database, the most similar protein was Acr2p. As discussed in chapter 1, Acr2p proteins share sequence similarities (including the essential HCXXXXXR motif) with Cdc25a protein tyrosine phosphatases, a separate class of phosphatases from the pI258 ArsC-related low molecular weight tyrosine phosphatases. This motif was also found in LmjF32.2740: His176, Cys177 and Arg183, respectively. Meanwhile the Cdc25a protein GXGXXG motif required for phosphatase activity is absent in both the *L. major* and *S. cerevisiae* Acr2p proteins.

LmjF32.2740 has recently been characterised and exhibits arsenate reductase activity *in vitro* (Zhou *et al.*, 2004); the protein has been designated LmACR2. In addition when LmACR2 was over-expressed in *L. infantum* the transgenic parasites displayed increased sensitivity to Pentostam; a similar phenotype was also observed when the *Leishmania* gene was expressed in *E. coli*. Although it appears that LmACR2 is the first example of an arsenate reductase in *Leishmania*, the protein that has been characterised is significantly shorter than LmjF32.2740. The possible reasons for this, and the potential implications, are discussed in section 1.5 of this chapter.

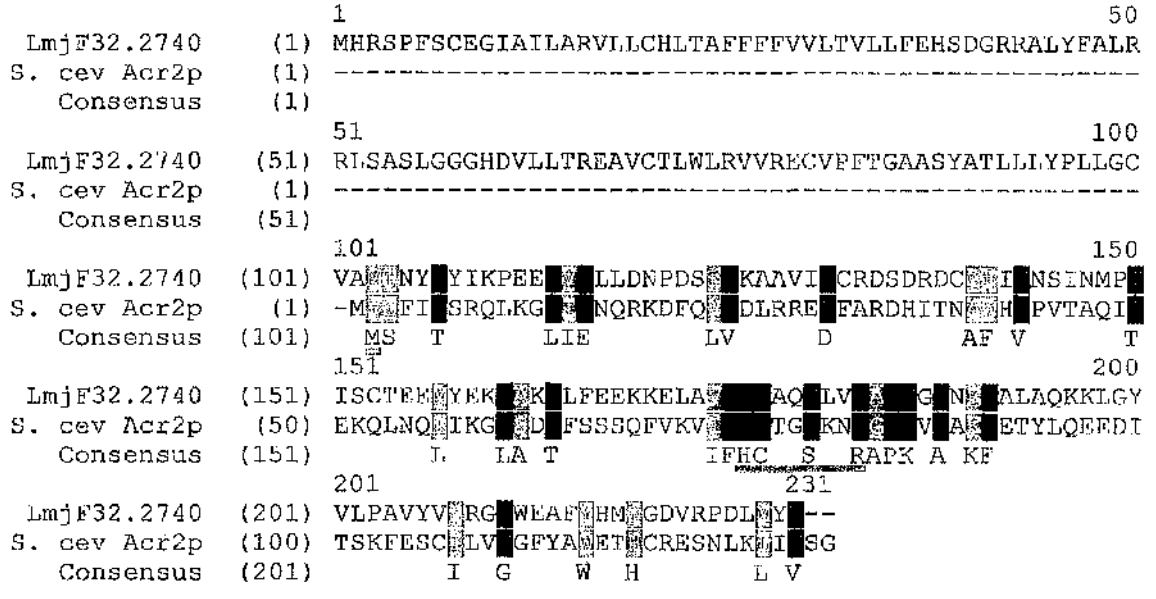


Figure 3.3: Alignment of *S. cerevisiae* Acr2p with the most similar *L. major* sequence, LmjF32.2740. S. cev Acr2p *S. cerevisiae* Acr2p. The motif known to be important in the *S. cerevisiae* protein which is also preset in the *L. major* sequence is underlined in red. The residue that has previously been reported as the start methionine of the *Leishmania* protein (Zhou et al. 2004) is underlined in blue. Dashes indicate gaps in the alignment. Identical residues are printed in white on a black background and similar residues are printed in black on a grey background.

3.1.2 Mammalian arsenate reductases

As detailed in chapter one, the two recently identified mammalian enzymes capable of reducing arsenate *in vitro* – purine nucleoside phosphorylase (PNP) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) – may not be responsible for *in vivo* arsenate reductase activity. It is therefore within this context that *Leishmania* proteins with similar sequences to the mammalian arsenate reductases are evaluated: even very similar proteins, likely to be present due to the highly conserved sequences of these enzymes due to their important cellular roles, are treated with caution.

3.1.2.1 PNP

The sequence of the 289 amino acid human PNP was used to search the *L. major* genome for similar predicted proteins. The most significant homologue – with a p/n score of $7.1e^{-8}$ – was that of LmjF05.0830, a predicted protein annotated as a methylthioadenosine phosphorylase (MTAP) although this is inferred from homology rather than experimental evidence. At 306 amino acids LmjF05.0830 is similar in size to human PNP and the proteins share 21% identity and 42% conservation. When LmjF05.0830 was used as an enquiry sequence in a blastp search of human proteins, nine sequences were more similar to the *Leishmania* protein than the PNP, with the most alike being a MTAP. LmjF05.0830 is considerably more similar to this class of phosphorylase than to PNP (figure 3.4): the identity between the human protein (identification number NP002442) and the *L. major* sequence is 34%. Accordingly, the annotation in the database describing LmjF05.0830 as a MTAP can probably be regarded as accurate as the amino acid sequences are so closely related. It is interesting to note that there are no predicted proteins annotated as, or displaying significant homology to, mammalian PNP included in the *L major* genome.

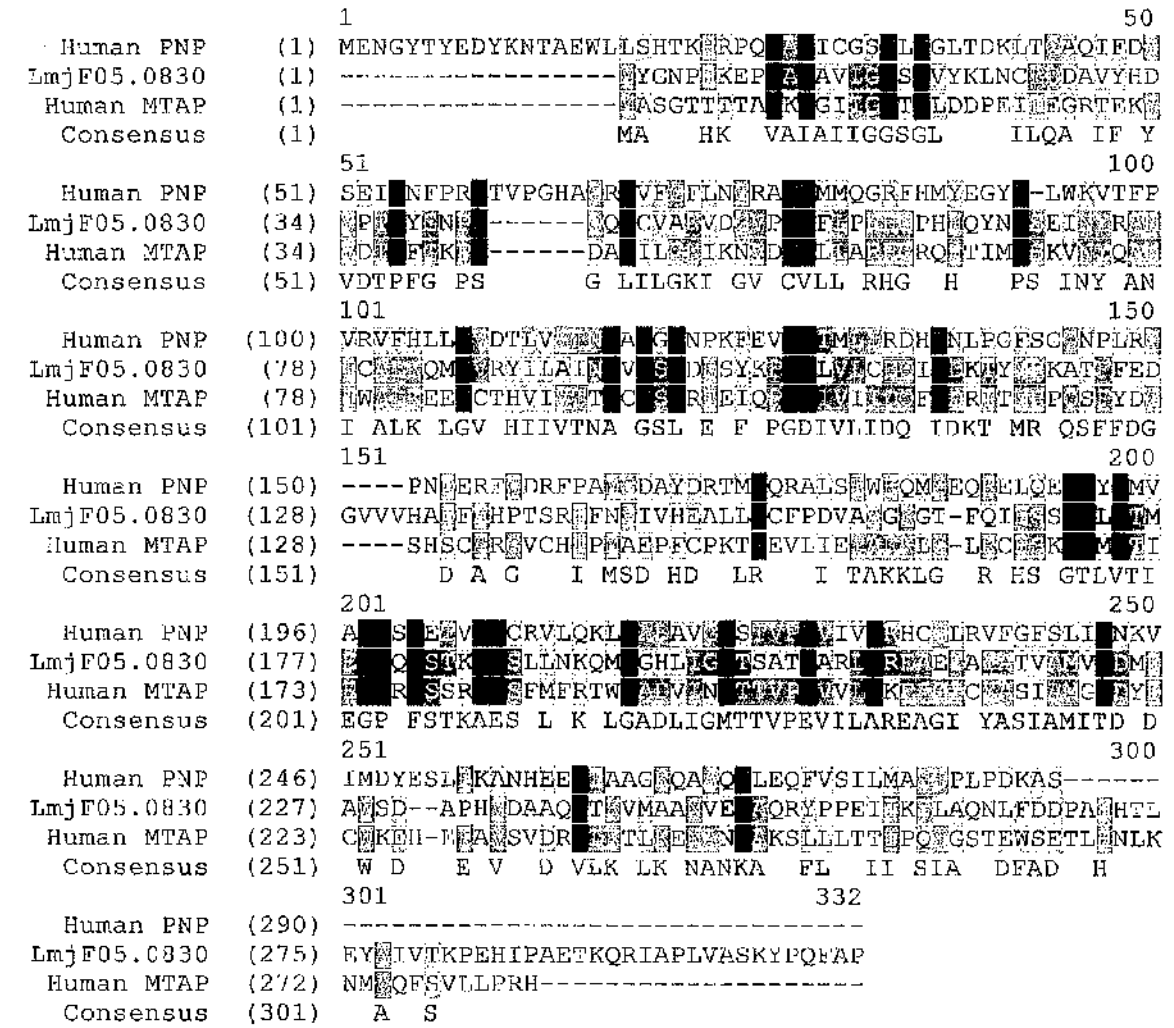


Figure 3.4: Alignment of Human PNP and MTAP sequences with the most similar *L. major* sequence, LmjF05.0830. The *L. major* sequence is more similar to human MTAP (identification number NP002442) than to PNP. Dashes indicate gaps in the alignment. Identical residues are printed in white on a black background, conserved residues are printed in white on a grey background and similar residues are printed in black on a grey background.

3.1.2.2 GAPDH

Using the sequence of the 335 amino acid human GAPDH protein as the inquiry sequence, the *L. major* genome was searched for similar proteins to this second class of mammalian putative arsenate reductases. Four *L. major* protein sequences were found to share significant similarity with human GAPDH and are all annotated as such. The first two of these four homologues, LmjF30.2970 and LmjF30.2980, will be considered together as they are tandemly arrayed and their sequences differ by just one amino acid. Both 361 amino acid proteins, which are similar in size to the human homologue, share 51% identity and 70% conservation with human GAPDH; these high values are reflected in the p/n scores which are $2.3e^{-86}$ for LmjF30.2970 and $5.3e^{-87}$ for LmjF30.2980. The predicted proteins are annotated as glycosomal GAPDHs and are >95% similar to an experimentally

characterised *L. mexicana* glycosomal GAPDH (Hannaert *et al.*, 1994). The glycosomal location of both these proteins together with the cytosolic location of the third *L. major* GAPDH has also been experimentally proven (Hannaert *et al.*, 1992).The third *L. major* GAPDH, LmjF36.2350, is just 215 amino acids long: over 120 amino acids present at the N-terminus of the human and other *L. major* GAPDH proteins are absent. However the p/n score is highly significant – $5.7e^{-74}$ – and the identity and conservation between the aligning regions of LmjF36.2350 and human GAPDH are 68% and 80%, respectively. The predicted protein, which is annotated as a cytosolic GAPDH, appears to be a partial GAPDH. However when the upstream DNA sequence was retrieved and translated, it too aligned with human GAPDH and proved to be the missing N-terminal part of the protein. A putative start methionine codon at the point in the sequence that would result in the missing portion being translated was also in frame, although a downstream stop codon prevented this sequence from being included in the LmjF36.2350 open reading frame. Whether a mutation has caused a stop codon to appear and for either a truncated protein to be translated or for LmjF36.2350 to have effectively become a pseudo-gene (perhaps possible due to degeneracy between the *L. major* GAPDH enzymes), or whether this is a straightforward error in the sequencing, is unclear. If the latter is true LmjF36.2350 is a strong contender to be a third *L. major* GAPDH and possible arsenate reductase. The fourth *L. major* GAPDH-like protein, the 349 amino acid Lmj35.4750, is less similar to the human enzyme with a p/n score of $9.3e^{-21}$. However this is a relatively significant value and the *Leishmania* sequence, which is annotated as a GAPDH, shares 26% identity and 45% conservation with the human protein. When each of the sequences of the *L. major* proteins similar to GAPDH were used as enquiry sequences in blastp searches of the human protein database, the most similar to each was GAPDH. All four *L. major* predicted GAPDH sequences aligned with the human GAPDH sequence are shown in figure 3.5.

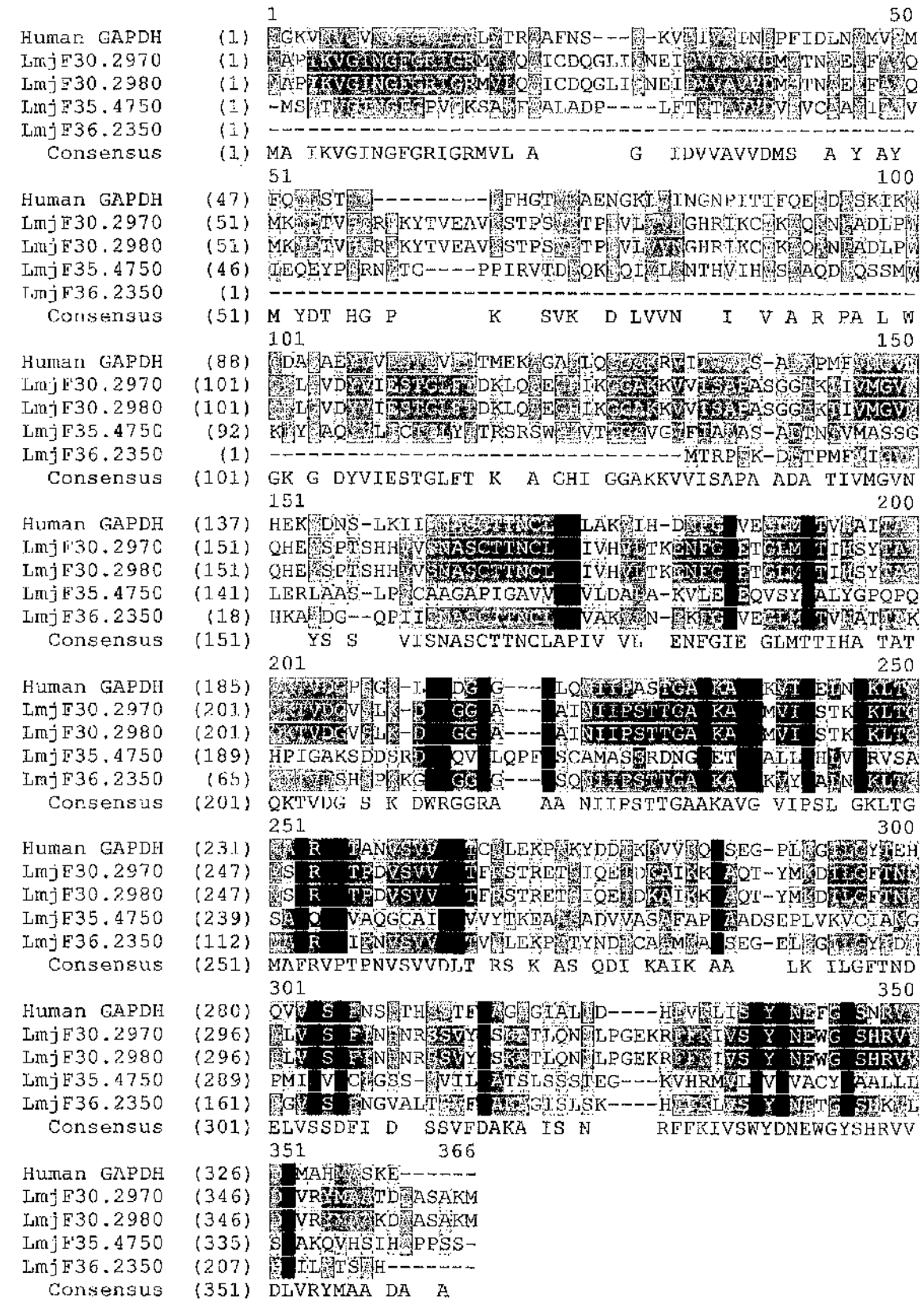


Figure 3.5: Alignment of human GAPDH with the most similar *L. major* sequences, LmjF30.2970, LmjF30.2980, LmjF35.4750 and LmjF36.2350. LmjF30.2970 and LmjF30.2980 are almost identical in sequence. Dashes indicate gaps in the alignment. Identical residues are printed in white on a black background, conserved residues are printed in white on a grey background and similar residues are printed in black on a grey background.

3.2 Analysis of omega glutathione S-transferase-like genes in *L. major*

In section 3.1, the presence in *L. major* of amino acid sequences similar to known arsenate reductase proteins was investigated. However, as is discussed in chapter one, reduction is not the only form of metalloid metabolism known to occur *in vivo*: biomethylation of arsenicals, and indeed antimonials, is also possible. Although most studies of biomethylation have focused on the methylation of arsenicals in mammalian systems (Zakharyan *et al.*, 1999), a similar process is known to occur in various microbes with antimonial species (reviewed in Bentley and Chasteen, 2002). The mammalian enzyme AS3MT is responsible for the methyltransferase activity (Lin *et al.*, 2002; Wood *et al.*, 2006). A search for similar enzymes to AS3MT in the *L. major* predicted proteins database yielded one significant (p/n score = $7.1e^{-05}$) homologue, LmjF35.4250, although the protein sequence is much more similar to, and is annotated as, 3-demethylubiquinone-9,3-methyltransferase, a methyltransferase involved in the synthesis of ubiquinone.

Meanwhile human enzyme MMA(V) reductase (which enzymatically reduces methylated pentavalent arsenicals as opposed to inorganic arsenate) has recently been identified as oGST (Zakharyan *et al.*, 2001). oGST sequences have since been identified in a wide range of organisms and have a conserved C-P-Y/F-A/V/S motif at their predicted active sites (figure 3.6). The role of oGST in arsenic metabolism *in vivo* remains unclear although naturally-occurring polymorphisms in *oGST* have been linked to arsenic-susceptibility in mammals (Schmuck *et al.*, 2005) and alterations in excreted arsenical profiles in humans (Marnell *et al.*, 2003). More recently oGST has been reported to reduce arsenate, as well as MMA(V), *in vitro* (Zakharyan *et al.*, 2005) – an observation that effectively adds a new class of proteins to the rapidly increasing number of arsenate reductases described earlier in this chapter. In this section an investigation of *L. major* genes and proteins similar to oGST is presented.

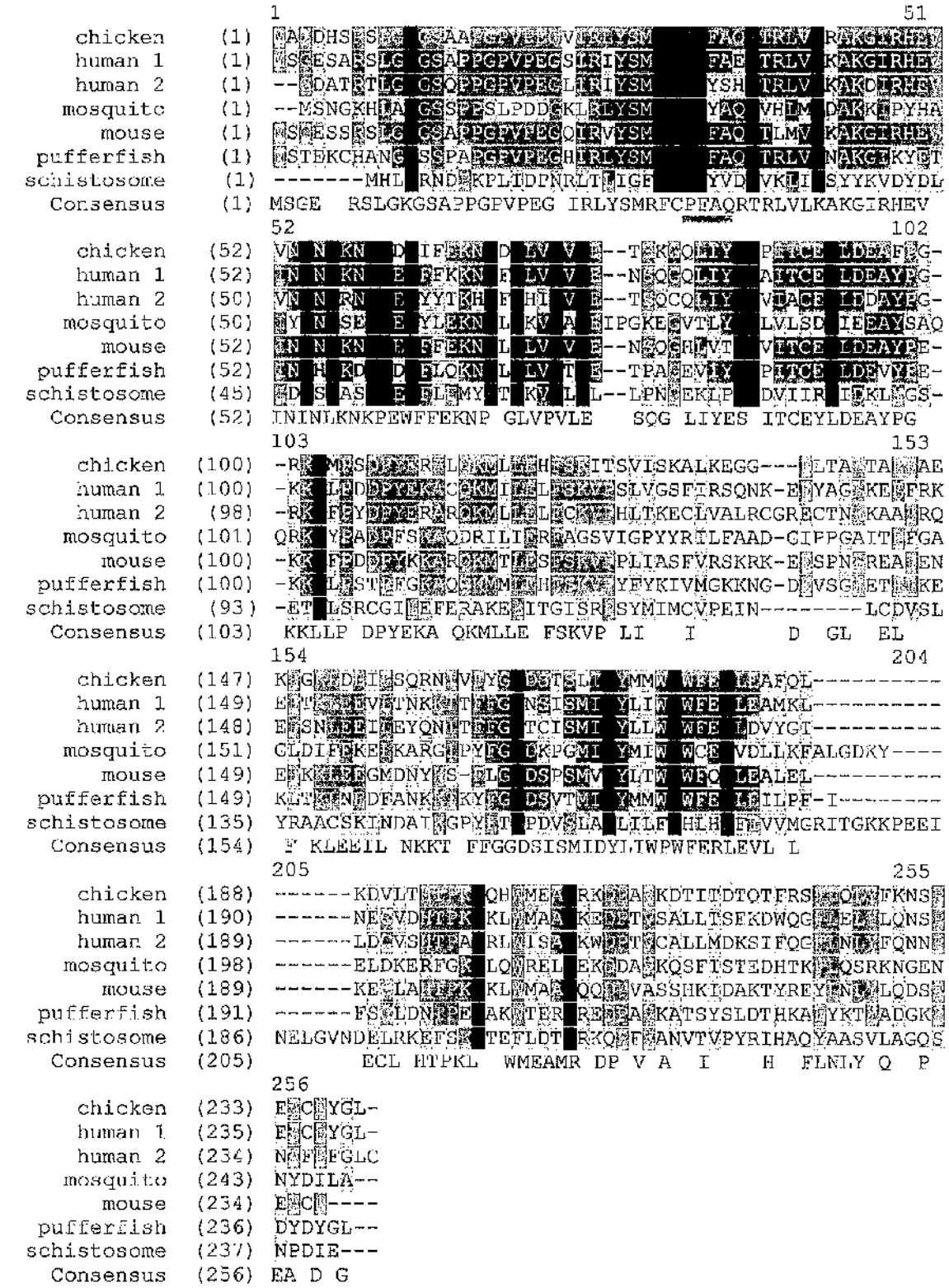


Figure 3.6: Alignment of various oGST amino acid sequences. oGST-like sequences have now been discovered in a diverse range of organisms. The sequences all share the predicted active site motif of C-P-Y/F-A/S/V situated at position 31-33 of human oGST1 (underlined in red). The identification numbers for the oGST sequences are as follows: chicken, XP421747; human 1, NP004823; human 2, NP899062; mosquito, AAP13482; mouse, NP034492; pufferfish, AAL08414; schistosome, AAO49385. Dashes indicate gaps in the alignment. Identical residues are printed in white on a black background, conserved residues are printed in white on a grey background and similar residues are printed in black on a grey background.

3.3 Omega GST-like sequences in the *L. major* genome

In order to identify *Leishmania* genes or proteins similar to oGST, the amino acid sequence of human oGST 1 (identification number NP004823) was used as an inquiry sequence to search the *L. major* databases available at www.genedb.org. This search was initially performed in 2003 when the *L. major* genome had not been fully sequenced. Several sequences, deemed lm16, lm33 and lm34 based on the *Leishmania* chromosomes they mapped to, displayed significant similarity to oGST, including in the active site motif (figure 3.7). As stated the sequencing of the genome at this time was incomplete and the databases not yet annotated; for each fragmentary oGST-like sequence the specific codon that translated to the start methionine was ambiguous. Furthermore lm34 lacked an in-frame stop codon. Unfortunately analysis of the surrounding sequences *in silico* to elucidate the open reading frames was impossible due to the non-assembled condition of the genome. Consequently it was necessary to perform rapid amplification of cDNA ends (RACE) on the sequences – 5' RACE on all three sequences and 3'RACE on lm34 – in an attempt to reveal the complete genes. This was successful for the sequence lm33, as detailed later in this chapter.

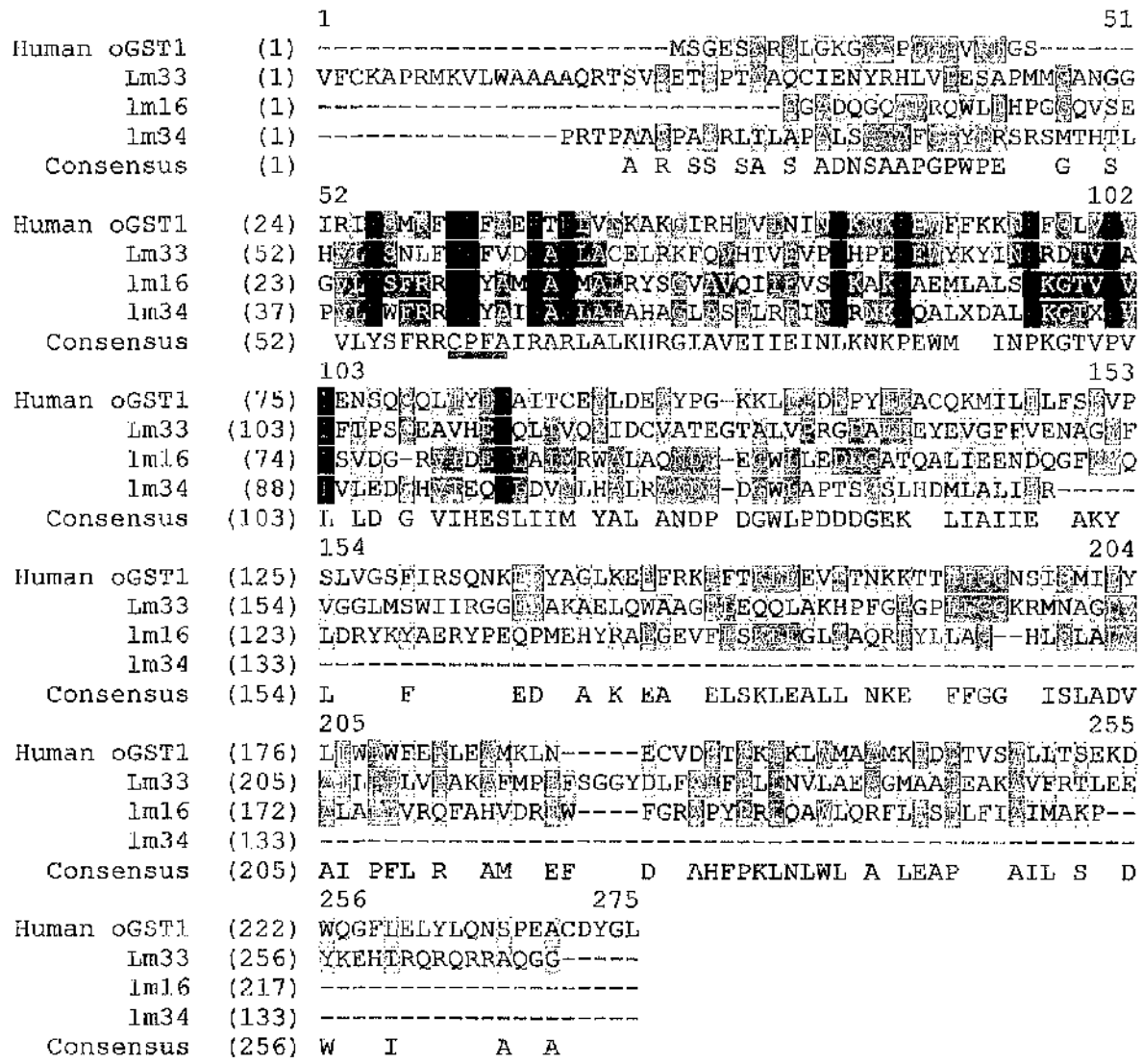


Figure 3.7: Alignment of *L. major* oGST-like sequences with human oGST1. All sequences are fragmentary: lm33 has a putative start methionine at position 8 but lm16 and lm34 do not, while lm34 also lacks an in-frame stop codon. However all sequences contain the conserved C-P-Y/F-A/V/S active site motif present in oGSTs (underlined in red). Dashes indicate gaps in the alignment. Identical residues are printed in white on a black background, conserved residues are printed in white on a grey background and similar residues are printed in black on a grey background.

3.3.1 *lm16* and *lm34*: analysis of two oGST-like sequence fragments

As mentioned, RACE was performed on the fragmentary lm16 and lm34 sequences to discover the positions of the relevant start and stop codons and hence elucidate the complete open reading frames. Frustratingly, despite repeated attempts, the reactions did not yield any products of the expected size; accordingly the DNA that was sequenced was found to be non-specific and unrelated to the sequences being analysed. Furthermore, attempts to amplify lm16 and lm34 by standard PCR also proved to be unsuccessful. At this point the sequences were used as inquiry sequences in tblastx searches of the translated

database at www.ncbi.nlm.nih.gov. Both sequences, particularly lm16, were found to share very high similarity with proteins from rod-forming, gram-negative bacteria *Photobacterium profundum* and *Pseudomonas fluorescens* (figure 3.8). This was unexpected as the sequences of oGST-like sequences are not particularly highly conserved between species (figure 3.6). The high level of similarity between the *Leishmania* and bacterial sequences, together with the experimental difficulties encountered when trying to amplify the genes, lead to the hypothesis that lm16 and lm34 may not be *L. major* genes and may have been included in the genome database due to bacterial contamination in the sequencing material. Therefore no further analysis of lm16 and lm34 was attempted at this time. Due to the fact that neither sequence appears in the now completed *L. major* genome, the hypothesis was likely to have been correct and the decision to terminate investigation into these sequences justified.

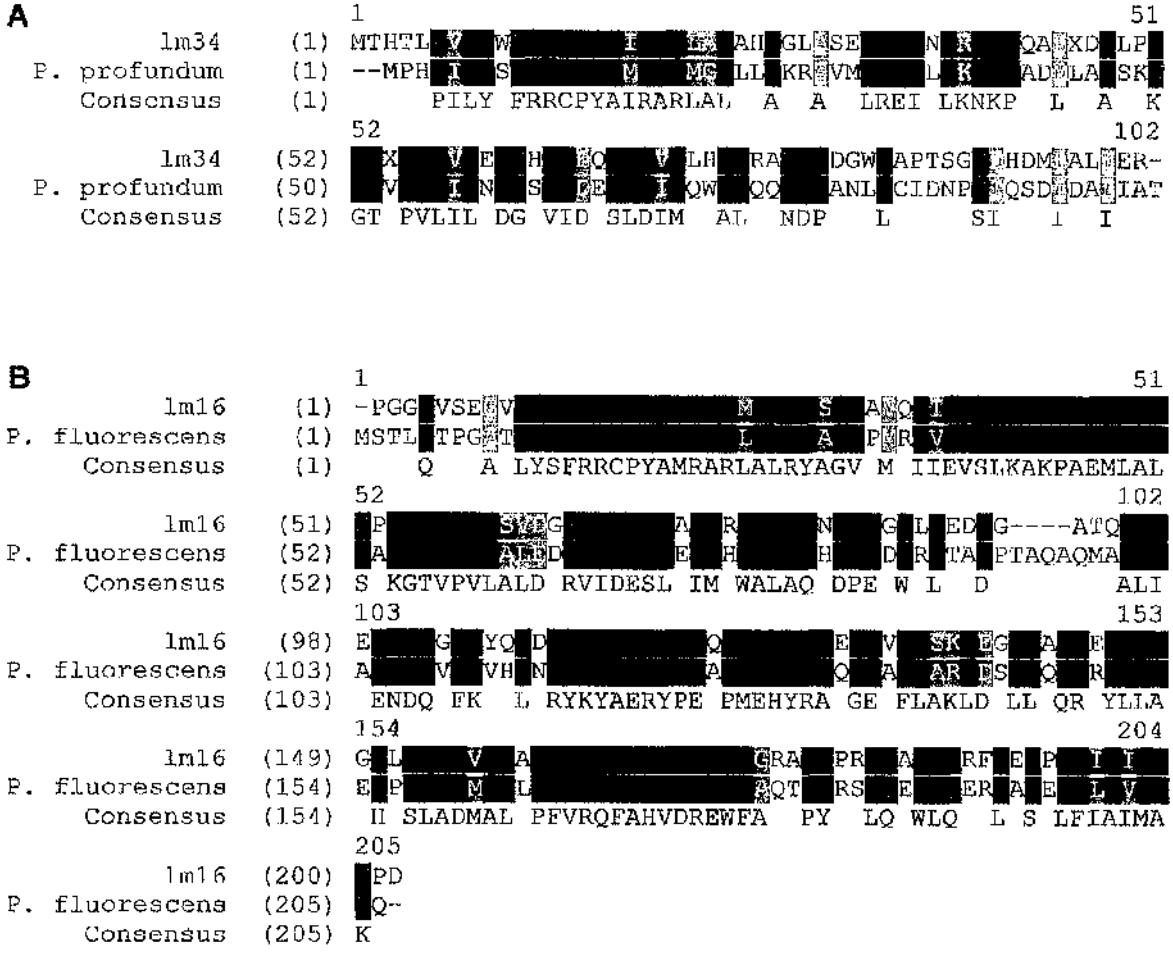


Figure 3.8: Alignment of lm16 and lm34 with bacterial amino acid sequences. A – lm34 aligned with *Photobacterium profundum* sequence. B – lm16 aligned with *Pseudomonas fluorescens* sequence. The high level of similarity coupled with the failure to amplify the supposed *Leishmania* sequences from parasite DNA suggested the lm16 and lm34 may be bacterial. The sequences no longer appear in the *L. major* genome database. Dashes indicate gaps in the alignment. Identical residues are printed in white on a black background and similar residues are printed in black on a grey background.

Genes annotated as glutaredoxins and thioredoxins in the *L. major* genome

Previously oGST has been shown to share amino acid sequence similarity (including the proposed active site motif), and enzymatic activities with glutaredoxins and thioredoxins (Board *et al.*, 2000) and structural identity with *E. coli* glutaredoxin 2 (Xia *et al.*, 2001). Indeed recombinant oGST is more similar to glutaredoxins in activity than to other classes of GSTs. Whether glutaredoxins or thioredoxins are also capable of directly reducing pentavalent metalloids – either inorganic or methylated – has never been explored. Such an activity could explain the previous observation that the transformation of arsenite to dimethylarsinate, which involves the reduction of methylarsonate, was possible *in vitro* with just the arsenical-methylating protein AS3MT, thioredoxin, thioredoxin reductase and NADPH present (Thomas *et al.*, 2004). The likenesses between methylarsonate-reducing oGST and these proteins, coupled with the possibility that they may directly reduce arsenicals, was the basis for analysing the *L. major* predicted proteins database for glutaredoxins and thioredoxins.

There are five *L .major* predicted proteins annotated as being putative glutaredoxins although in all cases the function has been inferred from homology rather than being based on experimental analysis. They are LmjF14.1480 (311 amino acids), LmjF20.1010 (107 amino acids), LmjF05.0310 (180 amino acids), LmjF27.0810 (109 amino acids), and LmjF01.0110 (195 amino acids). The largest of these predicted proteins, LmjF14.1480 is annotated as being a putative glutathione S-transferase or glutaredoxin. Two *L. major* predicted proteins, LmjF01.0270 and LmjF35.1250, are also annotated as being putative thioredoxins (again, function inferred from homology). When the amino acid sequences of these predicted proteins were used as inquiry sequences for blastx (translated sequence versus protein database) searches at www.ncbi.nlm.nih.gov, all were most similar to predicted or experimentally characterised glutaredoxins or thioredoxins, again with the exception of LmjF14.1480 which was most similar to prostaglandin E synthase 2. These proteins have been previously found to possess glutathione S-transferase activity; accordingly LmjF14.1480 is treated as such here and is discussed in section 3.4 of this chapter.

The *L. major* amino acid sequences were compared to those of glutaredoxins 1-5 (grx1-5) and thioredoxins 1 and 2 (trx1 and trx2) of the fission yeast *Schizosaccharomyces pombe*, and aligned using alignX (figures 3.9 and 3.10). The thioredoxin-specific motif WCGPCK is present in just one of the *L. major* predicted thioredoxins, LmjF01.0270, and this protein

also shares the highest level of sequence identity with trx1 and trx2 of *S. pombe*. Even more strikingly, LmjF01.0270 is 56% identical to *T. brucei brucei* thioredoxin which has been experimentally characterised (Reckenfelderbaumer *et al.*, 2000). However, the second predicted thioredoxin, LmjF35.1250, does not contain the aforementioned motif but does possess a comparable N-terminal WCEPCT sequence (figure 3.9). In addition the predicted protein has a CGFT motif which is similar to, and aligns with, the CGFS active site motif of the *S. pombe* glutaredoxins grx4 and grx5 (figure 3.10). *S. pombe* grx4 also contains an N-terminal thioredoxin-like motif, WAAPCK, potentially responsible for cellular nuclear localisation rather than thioredoxin or glutaredoxin-like activities (Molina *et al.*, 2004). LmjF35.1250 shares considerably higher levels of identity with grx4 and grx5 – 20% and 28%, respectively – than with trx1 (11%) and trx2 (8%). It is reasonable to conclude that LmjF35.1250 is a monothiol glutaredoxin that contains a thioredoxin-like motif, rather than an actual thioredoxin protein.

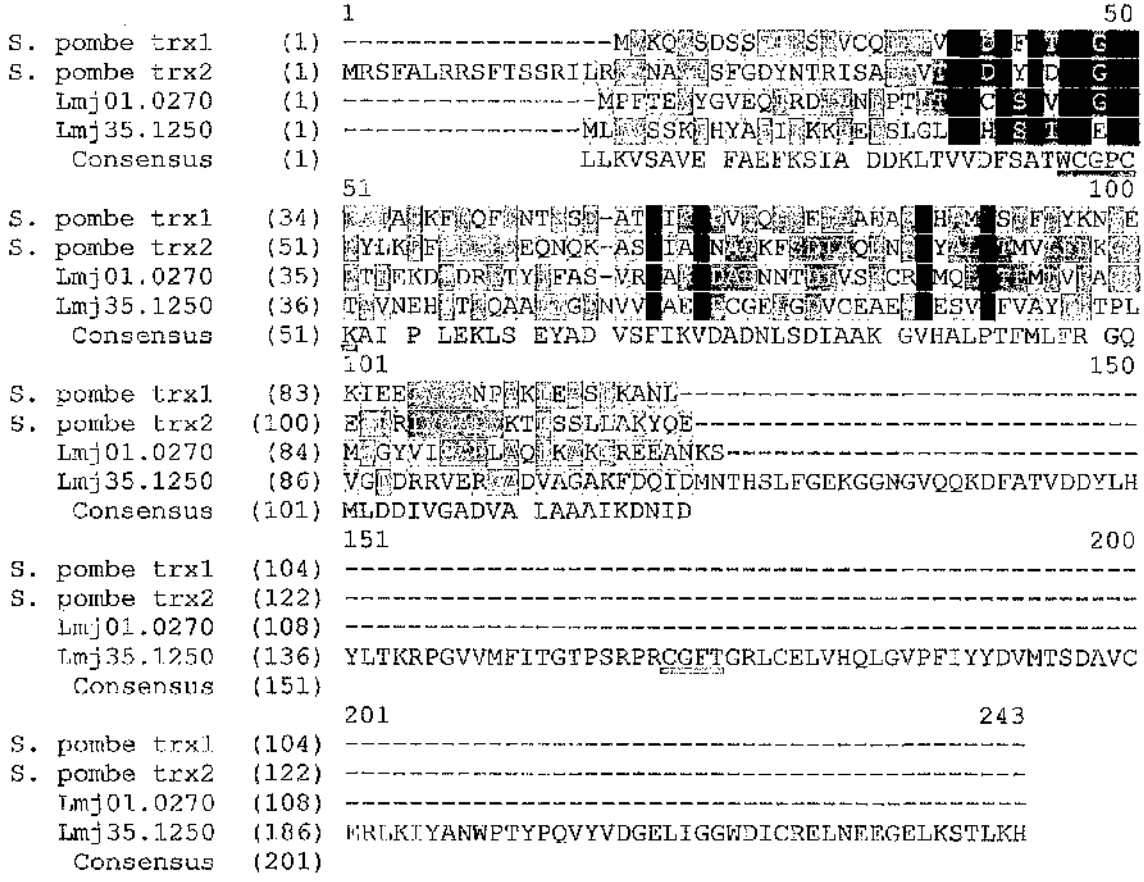


Figure 3.9: Alignment of S. pombe trx1 and trx2 with the annotated *L. major* trx sequences, LmjF01.0270 and LmjF35.1250. The trx-specific motif is present in LmjF01.0270 but not LmjF35.1250 (underlined in red). The grx-like motif in LmjF35.1250 is underlined in blue. Dashes indicate gaps in the alignment. Identical residues are printed in white on a black background, conserved residues are printed in white on a grey background and similar residues are printed in black on a grey background.

Of the annotated glutaredoxins (with the exception of LmjF14.1480), all aligned with *S. pombe* glutaredoxins and had similar active site motifs to them (figure 3.10). Only one, LmjF27.0810, had a conventional glutaredoxin dithiol active site (C-P-Y-C). This protein also shared highest levels of identity, in both cases 35%, with the *S. pombe* dithiol glutaredoxins grx1 and grx2. The predicted active sites of the remaining *L. major* putative glutaredoxins contain just one cysteine: C-A-F-S (LmjF01.0110), C-R-F-T (LmjF05.0310) and C-Q-F-S (LmjF20.1010). LmjF20.1010 shares 24% sequence identity with both grx4 and grx5 but the aligned sequence is 28% identical to grx1. However, because of the single cysteine residue in the active site motif of these *L. major* predicted proteins, all three of them are likely to be monothiol glutaredoxins. Two of them, LmjF01.0110 and LmjF05.0310, are predicted to have N-terminal signalling tags and when the amino acid sequences were entered into targetP they were both predicted to be mitochondrial proteins.

In summary, *L. major* is likely to contain one thioredoxin, one dithiol glutaredoxin and four monothiol glutaredoxins, up to three of which may localise to cellular organelles, such as the mitochondria, or be secretory proteins. Although their primary role is unlikely to be metabolising metalloids in the parasite, investigation into whether any of these predicted proteins are capable of reducing and hence activating pentavalent antimonial drugs would be worthwhile. However, these have not been analysed in this study due, in part, to the presence of sequences sharing considerable similarity to those of the verified mammalian arsenical reductase oGST in the *L. major* genome, as described previously.

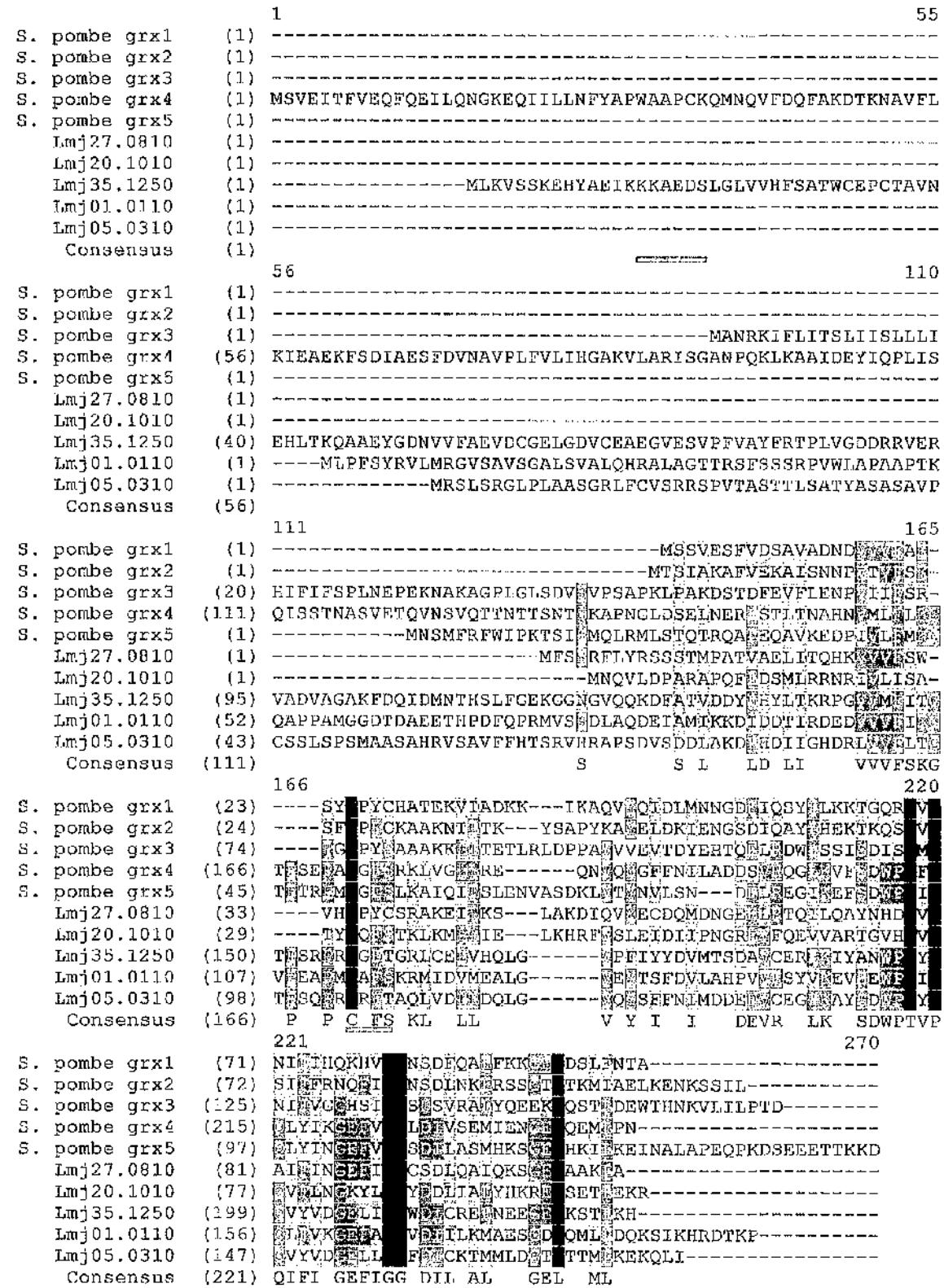


Figure 3.10: Alignment of *S. pombe* grx1, grx2, grx3, grx4 and grx5 with the annotated *L. major* grx sequences, LmjF27.0810, LmjF20.1010, LmjF01.0110 and LmjF05.0310; plus LmjF35.1250. The trx-like motif present in *S. pombe* grx4 is underlined in red. The predicted grx active sites, including monothiol and dithiol motifs, are underlined in blue. Dashes indicate gaps in the alignment. Identical residues are printed in white on a black background, conserved residues are printed in white on a grey background and similar residues are printed in black on a grey background.

3.4 Analysis of S-hexyl-GSH-binding proteins in *Leishmania*

Glutathione S-transferases (GSTs) are a diverse family of detoxification enzymes present in most life forms that catalyse the conjugation of glutathione (GSH) to both endogenous and exogenous electrophillic compounds. They have been implicated in a wide range of cellular reactions (Hayes *et al.*, 2005) including drug-resistance, notably to anti-cancer compounds (Townsend and Tew, 2003). It is thus possible that GSTs could be involved in directly detoxifying antimonial compounds. However, the recently discovered involvement of mammalian oGST in arsenical reduction (Zakharyan *et al.*, 2001) was the most compelling reason to look for GSTs in *Leishmania*.

In 2002 several known GST sequences were used as inquiry sequences in searches of the various *L. major* genome and proteome databases. With the exception of the oGST-like sequences described previously, and with LmjF14.1480 being more similar to prostaglandin E2 synthase, there were no highly significant similar sequences to any of the classes of GST used in the searches. Prostaglandin E2 synthases are members of the MAPEG (Membrane Associated Proteins in Eicosanoid and Glutathione metabolism) family which include microsomal, membrane-bound GST's. Many of these proteins catalyse the conjugation of 1-chloro-2,4-dinitrobenzene with GSH (Bresell *et al.*, 2005), generally considered as being the standard GST activity. Therefore it is plausible that LmjF14.1480 could function as a cellular GST and, despite the associated problems in analysing membrane-bound proteins, could be an interesting candidate for investigation. However, *L major* sequences encoding cytosolic GSTs (like oGST) remained elusive. The abundance of cytosolic GSTs in most life-forms suggested that GST-encoding genes would be present in the *L. major* genome; the lack of them found at this time was explained by the difficulty in identifying GSTs due to the highly divergent nature of proteins belonging to this large, diverse super-family (Sheehan *et al.*, 2001). Therefore attempts were made to isolate GST proteins directly from *Leishmania* parasites using biochemical methods.

3.4.1 Isolating *Leishmania* proteins using S-hexyl-GSH sepharose

It has been reported that pig oGST binds to S-hexyl-glutathione (S-hexyl-GSH) and that this property can be exploited to purify the protein from cellular extracts (Rouimi *et al.*, 2001). This approach, together with mass-spectrometric analysis of the recovered proteins, was used here in an attempt to find novel *Leishmania* GSTs. S-hexyl-GSH sepharose was used as oGSTS were of specific interest due to their *in vitro* arsenate reductase activities.

Briefly, *Leishmania* parasites were harvested and lysed and the soluble fraction was incubated with S-hexyl-GSH. Bound proteins were subsequently eluted from the sepharose and analysed by SDS-PAGE; consistently appearing bands were analysed by mass-spectrometry so their identity could be ascertained.

3.4.2 The range of *Leishmania* proteins isolated using S-hexyl-GSH sepharose is reproducible

Before pursuing the identity of S-hexyl-GSH binding-proteins by mass spectrometric analysis it was important to optimise the elution conditions to enhance yield and reproducibility. Large cultures of *Leishmania* promastigotes were harvested so that each sample contained at least 1 x 10^9 parasites, a volume sufficient for the resultant eluted proteins analysed by SDS-PAGE to be visible following silver-staining. Both the amount of S-hexyl-GSH used in the elution buffer and the pH of the elution buffer were varied in order to determine whether bound proteins could be eluted gradually from the sepharose (figure 3.11A and 3.11B). However, it was found that this approach yielded similar protein compositions in each elution and therefore the number of elutions performed in subsequent experiments was limited. Using large volumes of parasites and silver-staining the SDS-PAGE gels did, however, provide confirmation that reproducibility in proteins eluted between different samples could be achieved (figure 3.11A). Several proteins were eluted in the range of 20-30 kD (figure 3.11B): these were of particular interest as cytosolic GSTs from other organisms are around this size.

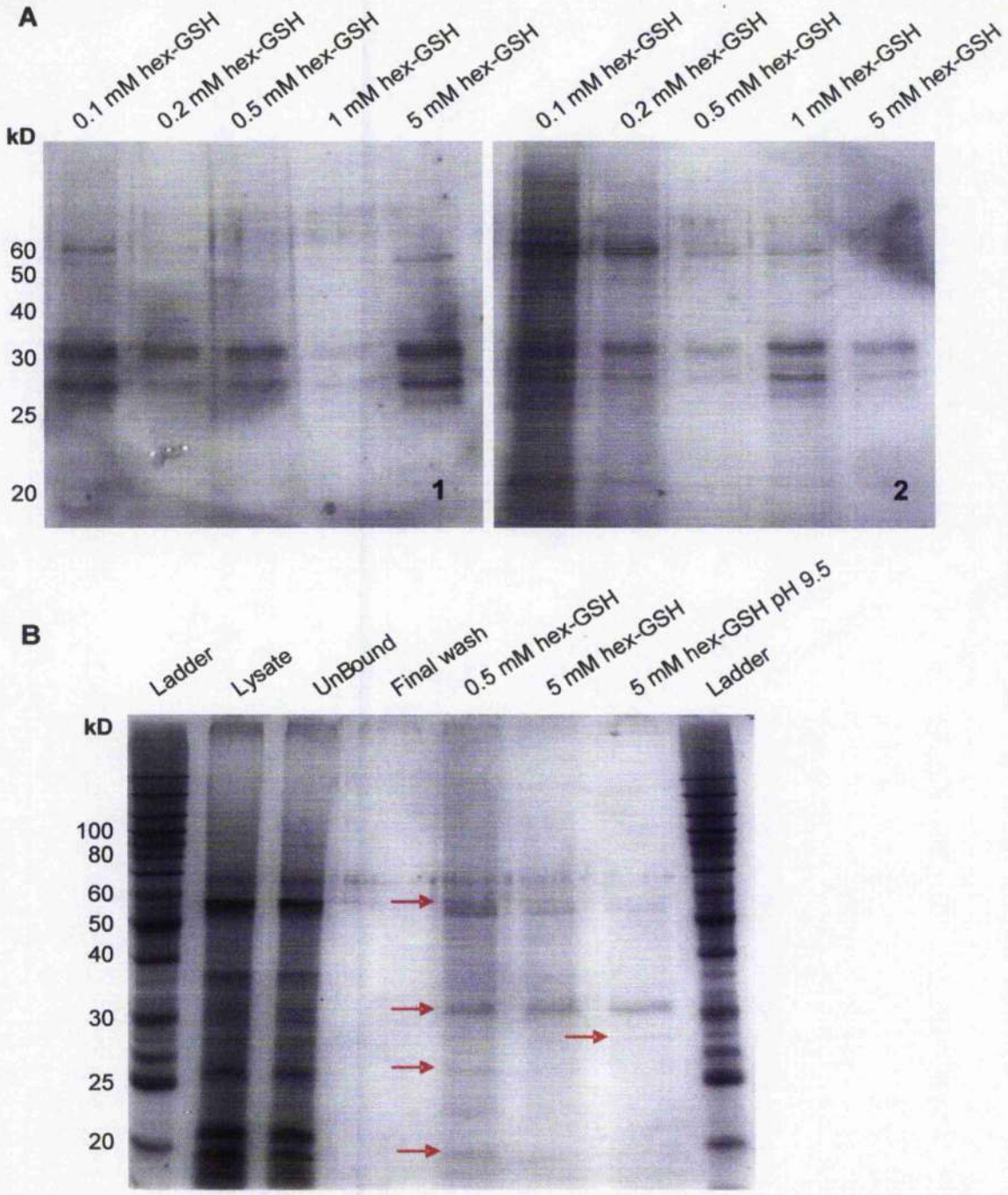


Figure 3.11: Silver-stained SDS-PAGE gels of *Leishmania* proteins eluted from S-hexyl-GSH sepharose. A1 and A2 – *L. infantum* proteins eluted with increasing concentrations of S-hexyl-GSH as labelled above the gel images. hex-GSH = S-hexyl-GSH. A1, 1.8 x 10^9 parasites used; A2, 2.6 x 10^9 parasites used. All elutions were performed in 50 mM Tris-HCl, pH 8.0. Protein molecular masses are indicated to the left of the gel image in kD (kilodaltons). B – *L. major* proteins eluted with different S-hexyl-GSH concentrations and high pH buffer, as labelled above as before. Final wash performed in bind/wash buffer pH 7.3. All elutions performed in Tris-HCl pH 8.0 except for final elution performed in same buffer but at pH 9.5 as indicated. Ladder shown is benchmark protein ladder; protein sizes are shown as before. Proteins eluted from the S-hexyl-GSH sepharose are indicated with red arrows.

3.4.3 Similar proteins are isolated from different species of *Leishmania* with S-hexyl-GSH sepharose

As well as achieving reproducibility between S-hexyl-GSH binding-proteins recovered from different *L. major* samples, it was desirable to ascertain whether similar proteins could be detected from different *Leishmania* species. A final wash with elution buffer without S-hexyl-GSH was introduced that was performed with the same volume as the elutions. This was to ensure that the proteins detected were being eluted specifically by the S-hexyl-GSH in the buffer, rather than by a non-specific effect due to the change in buffer and pH: proteins that were either only present or were more abundant in the elution samples were of interest as they were being specifically eluted by the S-hexyl-GSH. In each species – *L. major*, *L. infantum* and *L. mexicana* – one or two proteins were identified that met these criteria (figure 3.12). Moreover the proteins detected were of comparable sizes suggesting that the same proteins could be isolated from different *Leishmania* species using S-hexyl-GSH sepharose. A protein of approximately 30 kD, similar in size to oGST, was isolated from both *L. major* and *L. infantum*.

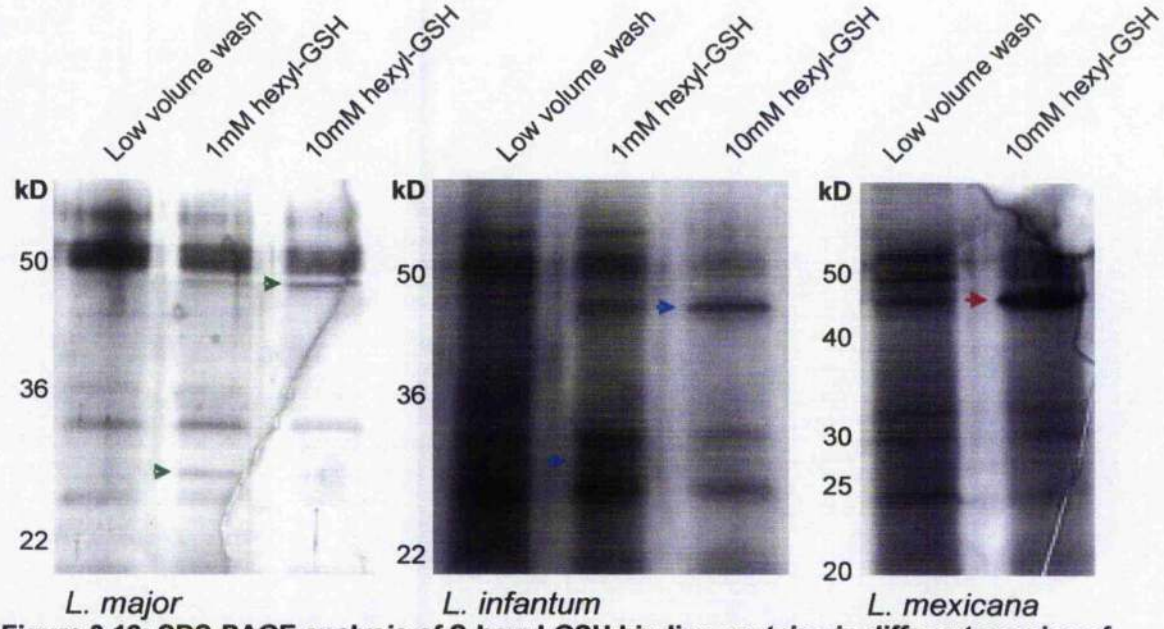


Figure 3.12: SDS-PAGE analysis of S-hexyl-GSH binding-proteins in different species of *Leishmania*. For each different species (as labelled below the gel images) the proteins in the final, low-volume wash and either one or two different elutions have been subjected to SDS-PAGE; the proteins have been visualised by silver-staining. Protein molecular weights are indicated to the left of each gel image in kD (kilodaltons). Proteins more abundant in the eluted fractions than in the washes have been identified and marked with arrows: *L. major*, green arrows, *L. infantum*, blue arrows, *L. mexicana*, red arrow. Similarly sized proteins were detected in each species.

3.4.4 Identification of proteins isolated from *Leishmania* with S-hexyl-GSH sepharose

Using *L. infantum* parasites the experiment was repeated, again using low-volume washes with the different buffers so that proteins eluted by specifically by S-hexyl-GSH could be recognised. This time the gel was stained with coomassie rather than silver nitrate as the former is compatible with mass spectrometry while the latter is not. Following resolution by SDS-PAGE, three proteins were visible that were significantly more abundant in the eluate as compared to the final wash (figure 3.13). These protein bands were excised and the contents of the approximately 30 kD protein band were analysed by mass spectrometry. Unfortunately the proteins identified did not correspond to any of the putative GST sequences already identified by searching the *Leishmania* genome databases. The most significant match was to pyroline-5-carboxylate reductase which at 28.7 kD is similar in size to the band excised for analysis. Alpha-tubulin, a commonly found contaminant due to its abundance in the cell, was also detected. The failure of this technique to identify any GSTs in *Leishmania* was, with hindsight not entirely unexpected: aside from those predicted proteins mentioned elsewhere in this chapter, no GSTs exist within the now fully-sequenced *L. major* genome.

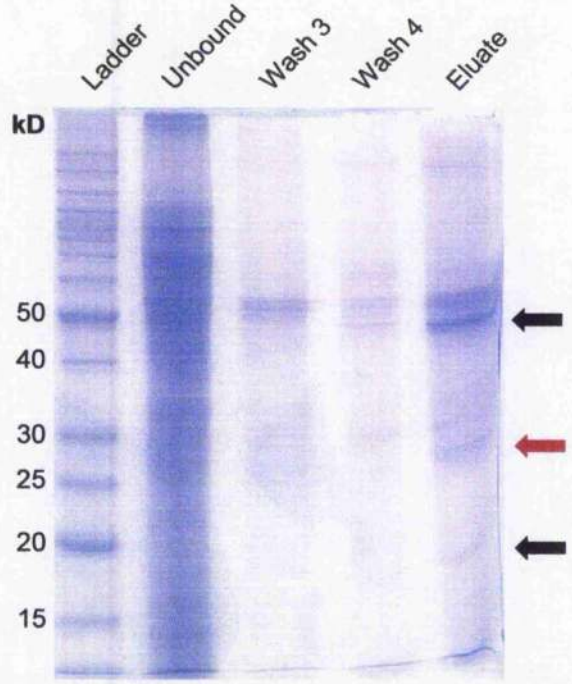


Figure 3.13: Coomassie-stained gel of *L. infantum* S-hexyl-GSH-binding proteins. The different fractions, as labelled above the gel image, have been subjected to SDS-PAGE analysis. Wash 3, low-volume wash at pH 7.5; wash 4, low-volume wash at pH 9.5; eluate, proteins eluted with 10 mM S-hexyl-GSH, pH 9.5. Protein molecular weights are indicated to the left of the gel image in kD (kilodaltons). Proteins that were more abundant in the eluate are marked with arrows. The red arrow indicates the ~30 kD band that was excised and analysed by mass spectrometry.

3.5 Identification and analysis of *TDR1* (*lm33*)

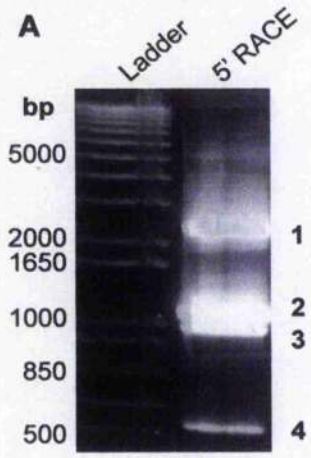
In section 3.3, the discovery of three oGST-like sequences in the *L. major* genome was described. Two of these – lm16 and lm34 – were shown to be probable bacterial sequences and no longer appear in the genome. Like lm16 and lm34 the third sequence – called lm33 after the chromosome the sequence was consigned to – was fragmentary and molecular analysis was required to elucidate the complete coding sequence. That investigation and subsequent identification of the gene is described in this section.

3.5.1 Identification of the complete *TDR1* gene

5'RACE was performed on the lm33 in order to locate the position of the start methionine. The sequence had originally been identified due to its similarity to human oGST and the complete gene was predicted to be of a similar size to the human sequence: approximately 723 nucleotides. The position of the internal primer determined that the 5'RACE reaction should yield a product of 200 base pairs plus the unknown 5' untranslated region, likely to be about another 100-200 base pairs. Unexpectedly, gel electrophoresis revealed four distinct products of approximately 450 base pairs, 950 base pairs, 1100 base pairs and 2200 base pairs (figure 3.14A). Although the smallest was most likely to be the desired product, all four bands were excised and ligated into the pGEM T-easy vector. Clones of the vector containing each of the three smallest products were obtained and the DNA was harvested and sequenced.

Surprisingly, sequencing revealed that the smallest product in the reaction was non-specific (did not contain any of the lm33 sequence) while both the 950 and 1100 base pair products contained the known sequence of lm33. Indeed, these two products transpired to be almost identical, differing only in the lengths of their untranslated N-terminal regions corresponding to two different transcripts. Both products contained approximately a further 625 base pairs of additional coding sequence as well as the 200 base pairs previously predicted. As a result the lm33 coding sequence contained a large N-terminal extension when compared to those of mammalian oGSTs; the complete gene was 1353 base pairs long as opposed to the 723 base pairs of human oGST1. The entire lm33 gene, including the variant 5' untranslated regions, and resultant amino acid sequence is shown in figure 3.14B. The gene was named *TDR1*, an acronym for thiol-dependant reductase 1, alluding to the activity of the protein it encodes. Analysis of the TDR1 amino acid sequence showed that the newly-revealed N-terminal half of the protein was also similar to oGST and consequently the two halves of TDR1 were similar to each other (figure 3.15).

A



B

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   1  AACTAACGCT ATATAAGTAT CAGTTTCTGT ACTTTATTGC CGAGCACCAC AGCAAGGAGG TCCCCTGCAG
  71  CCCTTTAGTG TCCGCTCCCC CTCGTCCACC TTCTTTTCCG TCTCGACTTG TTTTTCTGCT CTCCCACACG
 141  GGCTCTCACC GTCTGTGGCT CTCCCCCGTT AGAGAGGTGC GCCCGCTGCG TGTGTGTGTG TGCGTGTGTG
 211  TGTGTGTGTG TTGCTGCTGA AGCTTTCCGC TTATCTTGGC ACCGTTGGCC CACCACTGAA CCACTGAGTG
                       M   A   A   R   A   L   K   L   Y   V   S   A   T   C   P   F   C   H   R  ·
 281  CCTGGCGTGT CGGGGATGGC CGCGCGCGCG CTAAAGCTGT ACGTGTCGGC GACGTGCCCG TTCTGTCACC
      ·   V   E   I   V   A   R   E   K   Q   V   S   Y   D   R   V   A   V   G   L   R   E   E   M  ·
 351  GCGTGGAGAT CGTTGCGCGG GAGAAGCAGG TCTCCTACGA TCGGGTTGCT GTTGGGCTTC GCGAGGAGAT
      ·   P   Q   W   Y   K   Q   I   N   P   R   E   T   V   P   T   L   E   V   G   N   A   E   K
 421  GCCGCAATGG TACAAGCAGA TCAACCCGCG TGAGACAGTG CCGACGCTGG AGGTCGGCAA TGCGGAGAAG
       R   F   V   F   E   S   M   L   I   A   Q   Y   L   D   N   S   G   A   P   A   G   A   L   M  ·
 491  CGGTTTGTGT TCGAGTCGAT GCTGATCGCG CAGTACCTGG ACAACAGTGG CGCGCCCGCG GGTGCGCTGA
      ·   G   A   S   S   A   Q   R   H   Q   I   E   F   F   L   A   Q   V   G   D   F   I   A   A  ·
 561  TGGGTGCCTC GTCGGCGCAA CGACACCAGA TTGAGTTCTT CCTCGCCCAG GTCGGCGATT TCATTGCTGC
      ·   A   H   G   L   L   R   D   P   L   S   G   E   K   R   K   A   V   D   D   N   A   A   Y
 631  TGCGCACGGG CTACTCCGCG ACCCGCTGAG TGGTGAGAAG CGCAAGGCCG TGGATGACAA CGCGGCGTAC
       V   D   G   L   L   A   A   N   Q   T   T   G   P   Y   Y   C   D   G   E   F   T   M   A   D  ·
 701  GTGGACGGGC TGCTCGCGGC GAACCAGACG ACGGGGCCAT ACTACTGCGA CGGCGAGTTC ACGATGGCGG
      ·   V   A   L   V   P   F   L   V   R   L   K   P   A   L   M   Y   Y   A   G   Y   D   V   F  ·
 771  ACGTTGCGCT TGTGCCGTTC CTGGTGCGAC TGAAACCTGC TCTGATGTAC TACGCCGGGT ACGACGTGTT
      ·   C   K   A   P   R   M   K   V   L   W   A   A   A   A   Q   R   T   S   V   R   E   T   S
 841  CTGCAAGGCG CCACGAATGA AGGTGCTGTG GGCCGCTGCT GCTCAACGCA CATCTGTGCG TGAGACGTCG
       P   T   A   A   Q   C   I   E   N   Y   R   H   L   V   P   E   S   A   P   M   M   G   A   N  ·
 911  CCGACGGCGG CACAGTGCAT CGAGAACTAC CGCCACCTGG TTCCGGAGAG CGCGCCAATG ATGGGCGCCA
      ·   G   G   H   V   L   Y   S   N   L   F   C   P   F   V   D   R   A   R   L   A   C   E   L  ·
 981  ATGGCGGGCA CGTGCTGTAC AGCAATCTTT TCTGTCCTTT TGTGGACCGC GCACGCCTCG CGTGTGAGCT
      ·   R   K   F   Q   V   H   T   V   E   V   P   L   H   P   E   P   E   W   Y   K   Y   I   N
1051  GCGCAAGTTC CAGGTGCACA CGGTGGAGGT GCCGCTGCAT CCAGAGCCGG AGTGGTACAA ATATATCAAT
       P   R   D   T   V   P   A   L   F   T   P   S   G   E   A   V   H   E   S   Q   L   I   V   Q  ·
1121  CCCCGCGATA CGGTGCCTGC GCTATTTACG CCGAGCGGCG AGGCTGTACA CGAGTCGCAG CTGATTGTTC
      ·   Y   I   D   C   V   A   T   E   G   T   A   L   V   P   R   G   D   A   E   K   E   Y   E  ·
1191  AGTACATCGA CTGCGTGGCG ACGGAGGGTA CTGCGCTGGT GCCGCGTGGG GACGCGGAGA AGGAGTACGA
      ·   V   G   F   F   V   E   N   A   G   Y   F   V   G   G   L   M   S   W   I   I   R   G   G
1261  GGTGGGTTTC TTCGTGGAAA ACGCTGGGTA TTTCGTTGGA GGACTGATGT CGTGGATCAT CCGTGGTGGC
       E   D   A   K   A   E   L   Q   W   A   A   G   E   L   E   Q   Q   L   A   K   H   P   F   G  ·
1331  GAGGATGCGA AGGCTGAGCT TCAGTGGGCT GCTGGCGAGC TCGAGCAGCA GCTGGCGAAA CACCCGTTTG
      ·   E   G   P   F   F   G   G   K   R   M   N   A   G   D   V   A   I   L   P   F   L   V   R  ·
1401  GTGAGGGCCC CTTCTTCGGC GGCAAGAGGA TGAACGCTGG CGATGTGGCC ATTCTACCTT TCCTGGTCCG
      ·   A   K   A   F   M   P   E   F   S   G   G   Y   D   L   F   A   H   F   P   L   L   N   V
1471  TGCAAAGGCG TTTATGCCGG AGTTTAGTGG CGGGTACGAT CTCTTCGCCC ACTTTCCGCT GCTGAATGTG
       L   A   E   A   G   M   A   A   P   E   A   K   A   V   F   R   T   L   E   E   Y   K   E   H  ·
1541  CTGGCAGAGG CTGGCATGGC GGCACCGGAG GCGAAGGCGG TGTTTCGTAC ACTCGAGGAG TACAAGGAGC
      ·   I   R   Q   R   Q   R   R   A   Q   G   G   *
1611  ACATCCGTCA GCGTCAACGG AGGGCCCAGG GCGGGTAA
```

Figure 3.14: Elucidation of the complete *TDR1* (Im33) open reading frame. A – the amplified products of the 5' RACE reaction subjected to gel electrophoresis. Sizes in bp (base pairs) are indicated to the left of the image. Four distinct bands were visible, labelled 1-4, which were excised, cloned and sequenced. Bands 2 and 3 were the amplified Im33 transcripts. B – the Im33 (now called *TDR1*) open reading frame. The spliced-leader sequence is shown in red and the N-terminal untranslated sequence exclusive to the longer transcript (band 2 in part A of this figure) is shown in blue. The three letter amino acid codes are shown for the translated part of the sequence which encodes 450 amino acids rather than the 241 predicted. Where codons extend over two lines, dots are shown at the end and beginning of lines to represent this.

Figure 3.15: The N- and C- terminal halves of TDR1 are both similar to human oGST and to each other. Dashes indicate gaps in the alignment and the predicted active site motifs in both the N- and C- terminals are underlined in red. Identical residues are printed in white on a black background, conserved residues are printed in white on a grey background and similar residues are printed in black on a grey background.

Comparisons of the amino acid sequences using the vector NTI alignX function showed that human oGST shares 19% identity with the N-terminal half of TDR1 and 22% identity with the C-terminal half, while the halves are 28% identical to each other. It is interesting that despite their similarity, the two halves contain different predicted active site motifs: C-P-F-C in the N-terminal, and C-P-F-V in the C-terminal.

3.5.2 Tc52: Identification of a TDR1 homologue in *T. cruzi*

Using TDR1 as an inquiry sequence allowed detection of a similar sequence of comparable length in the *Trypanosoma cruzi* database (Tc00.1047053503419.30) and more recently in that of *L. infantum* (LinJ33.0270) (figure 3.16). When aligned as before, these sequences were shown to share 45% and 96% identity with TDR1, respectively. The predicted active site motifs are conserved in the N-terminal halves but the *T .cruzi* motif differs in the C-terminal: the cysteine residue at position 240 and the valine residue at position 243 in TDR1 are both replaced by serine residues in the *T. cruzi* protein. The *Leishmania* sequences are identical in these regions. Interestingly, no full-length sequences

homologous to TDR1 appear in the *T. brucei*, *T. vivax*, *T. congolense*, or *T. gambiense* predicted protein databases.

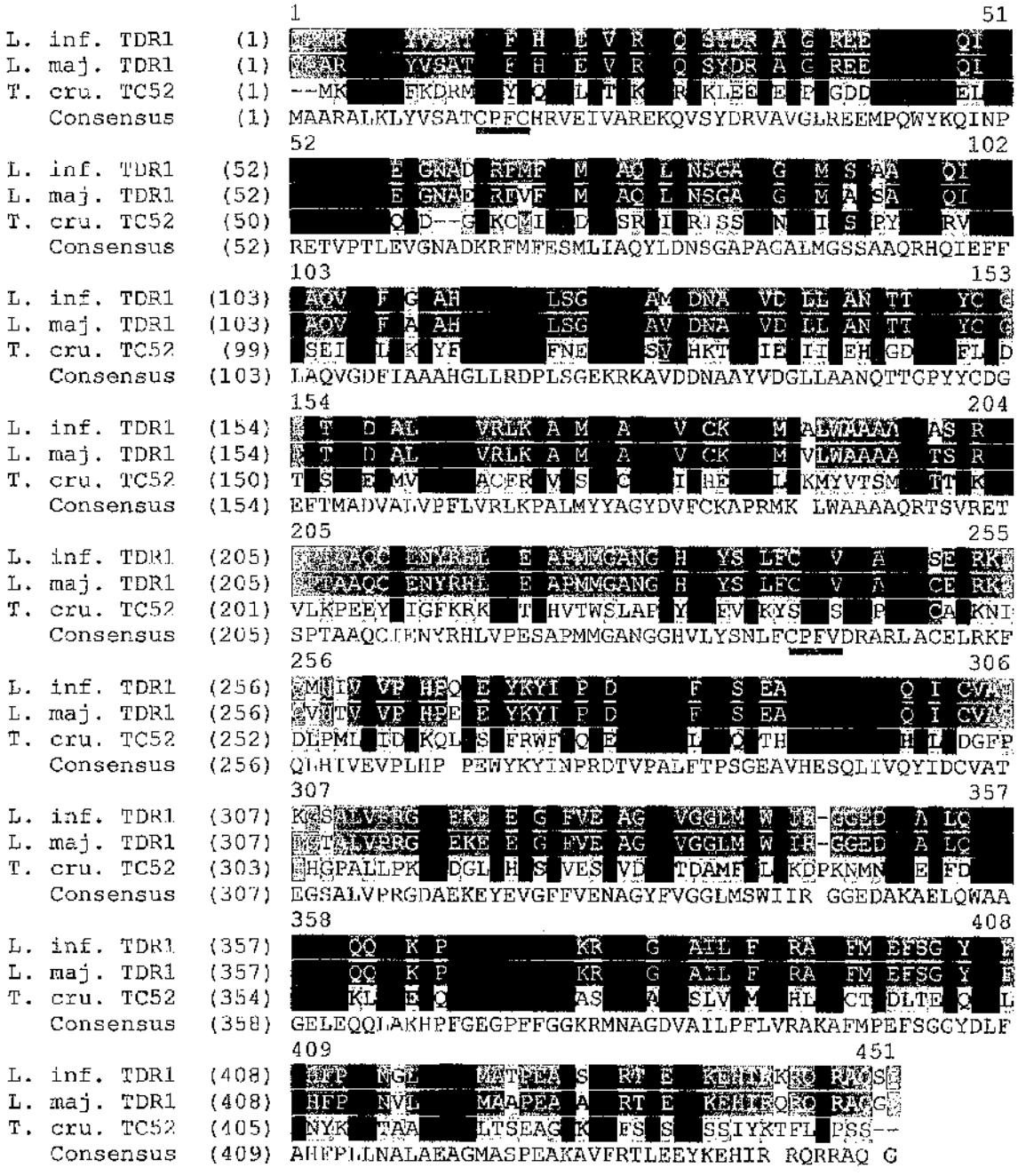

Figure 3.16: Alignment of TDR1 with similar sequences from *L. infantum* and *T. cruzi*. Dashes indicate gaps in the alignment and the predicted active site motifs in both the N- and C-terminals are underlined in red. Identical residues are printed in white on a black background, conserved residues are printed in white on a grey background and similar residues are printed in black on a grey background. L. inf. TDR1, *L. infantum* sequence (LinJ33.0270); L. maj. TDR1, *L. major* sequence (LmjF33.0240); T. cru. TC52, *T. cruzi* sequence (Tc00.1047053503419.30).

3.5.3 Amplification and cloning of *TDR1*

Before the complete TDR1 open reading frame (ORF) was elucidated, Dr. Helen Denton of the University of Glasgow had cloned the 3' half of the coding sequence into the pET28a(+) vector which allowed expression of the C-terminal region of TDR1 with an N-terminal His-tag. Therefore, following the discovery of the remainder of the gene, it was decided to clone both the 5' half of the coding sequence (5'TDR1) as well as the full-length TDR1 ORF. Both sequences were amplified from *L. major* genomic DNA (figure 3.17A) and sub-cloned into pGEM T-Easy before being cloned into pET28a(+) (represented in figure 3.17B). The production of recombinant TDR1 and 5'TDR1 is presented in chapter four.

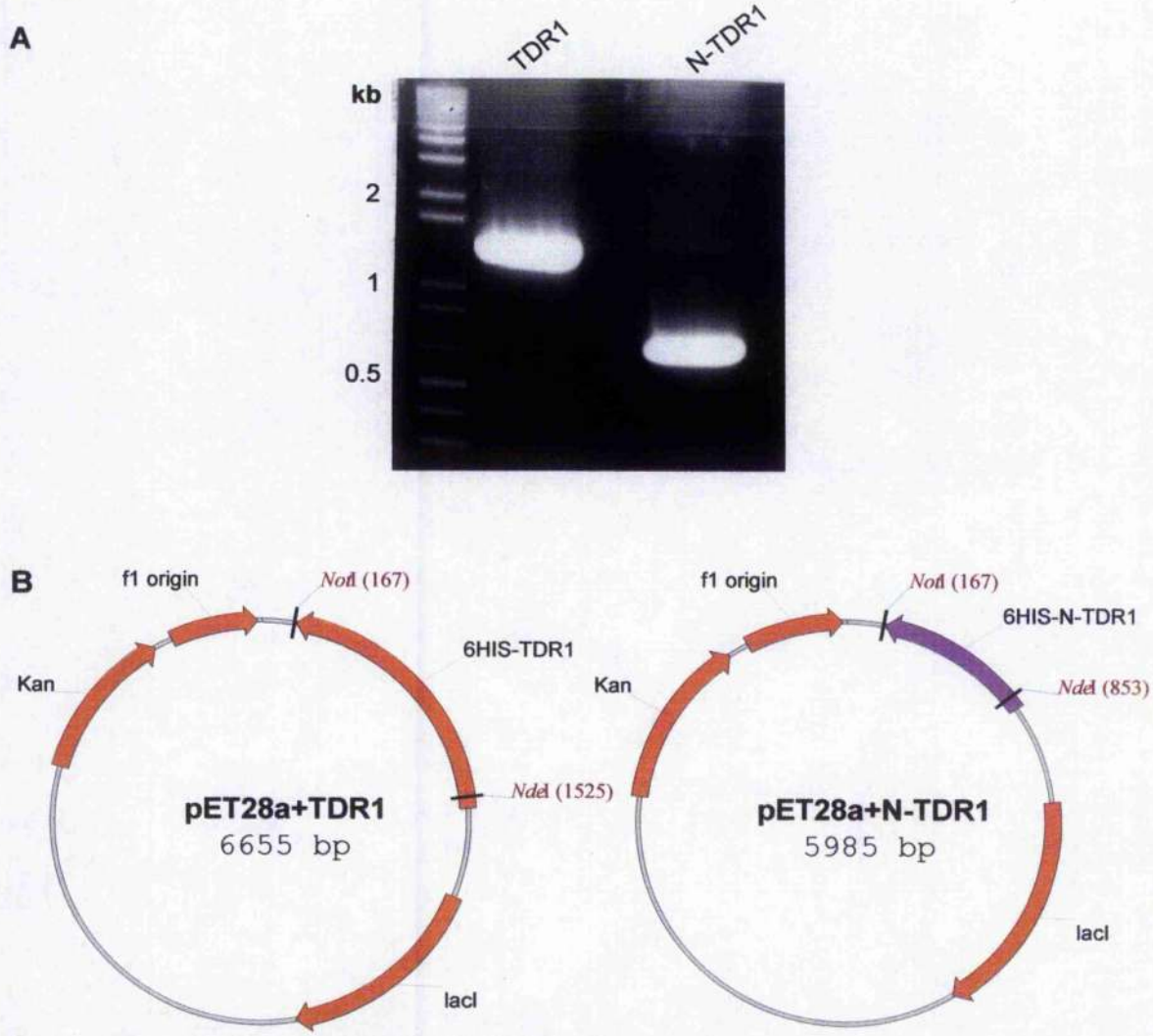

Figure 3.17: Amplification of TDR1 and 5'TDR1, and expression constructs for production of recombinant protein. A – ethidium bromide-stained gel showing the products of PCR amplification using primers JOFLOGST1 and JOFLOGST2 to amplify TDR1 and JOFLOGST1 and JOFLOGST3 to amplify N-TDR1, as labelled above the image. Sizes in kb (kilo bases) are indicted to the left of the image. B – schematic representation of the pET28a(+) plasmids constructed for the expression of TDR1 and N-TDR1. 6-HIS-TDR1/6-HIS-N-TDR1, the cloned ORFs with N-terminal His-tags; the restriction sites used to clone these into the vector are also shown. LacI, lac repressor gene; f1 origin, origin of replication of the f1 phage; kan, kanamycin resistance gene.

3.6 Discussion

In this chapter the most similar *Leishmania* proteins to different classes of known arsenical reductases have been elucidated and described. The three types of microbial arsenate reductases, which are well characterised in other organisms (Rosen, 2002), are not well represented in *Leishmania*; the most similar predicted proteins to both *S. aureus* pI258 ArsC and *E. coli* R773 ArsC are poor homologues of these proteins and lack residues essential for arsenate reductase activity. A more convincing potential arsenate reductase is LmjF32.2740, a *Leishmania* enzyme similar to *S. cerevisiae* Acr2p. This enzyme, which has been characterised and designated LmACR2, has been shown to reduce metalloids *in vitro* and increase sensitivity to pentavalent antimony when over-expressed in *L. infantum* (Zhou *et al.*, 2004). As described, the protein designated LmjF32.2740 in the geneDB database comprises 229 amino acids, 99 amino acids more than *S. cerevisiae* Acr2p; accordingly only 130 amino acids of the two proteins are similar and align. However Zhou *et al.*, reported that LmjF32.2740 (LmACR2) is just 128 amino acids long, ignoring the non-aligned 101 amino acid N-terminal extension. There is no published account of experimental evidence (5' RACE, western blots) that would confirm an error in the database of the size of the predicted protein. Furthermore, residues 13-35 of the complete 229 amino acid sequence of LmjF32.2740 are predicted to be a transmembrane targeting sequence and a possible cleavage site exists between residues 35 and 36. When the LmjF32.2740 amino acid sequence was entered into a protein localisation prediction programme, targetP, it was predicted to be a secretory protein. Clearly the predicted size of the LmjF32.2740 protein may be wrong: this could simply be an inaccuracy in the database and the protein may indeed comprise just 128 amino acids. However, the existence of the signal peptide near the beginning of the predicted 229 amino acid sequence suggests otherwise. Zhou *et al.* also found that LmACR2 is monomeric. This is in contrast to *S. cerevisiae* Acr2p which forms a homodimer and is suggestive of either a different catalytic mechanism or, as discussed, a discrepancy in the amino acid sequence of the recombinant protein.

If the complete LmACR2 protein does include an additional 101 amino acids at its N-terminal, there could be resulting implications for the published activities of LmACR2. It is possible that the full-length protein is more active and stable, possibly adopting a different quaternary structure from that of the monomeric formation found previously (Zhou *et al.*, 2004); indeed like *S. cerevisiae* Acr2p it may prove to be a dimer. In this case both the *in vitro* metalloid reductase activity of the recombinant protein and the degree of increased sensitivity to Pentostam achieved when the protein was over-expressed in *L.*

infantum may be improved by the inclusion of the additional residues. Conversely, if the wild-type protein is targeted to a particular organelle, is a membrane-embedded protein or, as predicted by targetP, secreted from the cell, it may be entirely unable to reduce pentavalent metalloids *in vivo*. Further experimental evidence is required to clarify the situation.

The absence of proteins significantly similar to bacterial arsenate reductases in *Leishmania* is perhaps unsurprising. These proteins, together with eukaryotic Acr2p, apparently arose separately by convergent evolution as a detoxification response to being exposed to arsenic (Mukhopadhyay and Rosen, 2002) and have no other known functions other than reducing arsenate. This is not the situation with recently identified mammalian proteins capable of reducing arsenate: oGST, PNP and GAPDH all display other enzymatic activities and their ability to reduce arsenate may be incidental.

For this reason the fact *L. major* does not appear to possess a protein likely to be a PNP was unexpected. PNPs catalyze the cleavage of ribonucleosides and deoxyribonucleosides, in the presence of inorganic orthophosphate (P_i) to generate the purine base and ribose(deoxyribose)-1-phosphate and in many organisms have a key role in the purine salvage pathway (Bzowska *et al.,* 2000). This pathway is essential in trypanosomatids as they are incapable of synthesising purines and instead salvage nucleosides and nucleobases from their host (reviewed in Landfear *et al.,* 2004). Despite blast searches not detecting any sequences similar to mammalian PNPs in *Leishmania*, a sequence annotated as a nucleoside phosphorylase-like protein is present in the *L. major* genome (LmjF10.1010). Analysis of this sequence showed that it was similar to the hexameric bacterial PNPs, which share function and topology with the trimeric PNPs found mainly in mammals, but are otherwise unrelated (Pugmire and Ealick, 2002). It is as yet unknown if bacterial PNPs can reduce arsenate *in vitro*; therefore whether LmjF10.1010 could potentially reduce and activate pentavalent antimonials drugs is also unclear.

As explained, the role of PNP and GAPDH in arsenate metabolism remains unclear. The situation is complicated by the fact that most of the *in vivo* work in regard to these enzymes as arsenate reductases has been performed on rats, the suitability of which has been questioned (Aposhian and Aposhian, 2006). However, the differences between arsenic metabolism in rats and humans are not necessarily relevant when assessing the role, if any, of these enzymes in unicellular *Leishmania*. If PNP does reduces metalloids *in vivo* it may contribute to their detoxification; therefore the absence of a similar protein in *Leishmania* could be related to their susceptibility to pentavalent antimonials. However

this is not in keeping with the findings of Shaked-Mishan *et al.*,, in which parasites incapable of reducing Pentostam were less, rather than more, sensitive to the drug. Likewise, if the arsenate reducing activity of GAPDH is shown to be relevant *in vivo*, antimonial susceptibility could be explained by the presence of several *Leishmania* GAPDH proteins metabolising pentavalent antimonials into more toxic trivalent forms. The situation will remain unclear until more is known about both the role of these proteins and how antimonials are toxic to *Leishmania.* Although the arsenate-reducing capacity of these mammalian enzymes observed *in vitro* has been described as 'fortuitous' (meaning the proteins have other functions and that the reducing activity may be coincidental and thus not relevant *in vivo*) (Waalkes and Liu, 2002) it is intriguing that potential metalloid reductases continue to be identified. Presumably the dynamic nature of the field of metalloid metabolism in other organisms reflects the situation in *Leishmania*: additional, as yet unidentified proteins may well be involved in antimonial reduction together with the ones examined here.

The most similar *L. major* proteins to all known classes of arsenate reductases have been described and the sequences analysed. Most of the proteins presented here are unlikely to function as arsenate reductases due to their low similarity to the enzymes including lack of essential residues or because they are more like other classes of proteins. Whether LmACR2, the *Leishmania* Acr2p capable of reducing pentavalent antimonials, has an N-terminal region previously unreported must be established for its *in vivo* role to be clarified. Of all the remaining predicted and known proteins described here the GAPDH homologues are perhaps the most promising as potential novel *Leishmania* arsenate reductases; the high level of conservation between their amino acid sequences and those of the mammalian enzymes may be indicative of a common function. However it is important that the arsenate reductase activity exhibited by mammalian GAPDH be verified *in vivo*. The fact that GAPDH was not implicated in metalloid metabolism until very recently, together with the continued uncertainty surrounding the true role of this enzyme in the reduction of arsenate, are the foremost reasons why the *L. major* enzymes were not investigated as part of this study.

At one time thioredoxins were thought to be absent from trypanosomatids due to their lack of thioredoxin reductase, required to reduce thioredoxin in other organisms. However the discovery and characterisation of a classical thioredoxin in *T. brucei brucei* (Reckenfelderbaumer *et al.*, 2000) which can be reduced directly by trypanothione (Krauth-Siegel and Schmidt, 2002) disproved this theory. The presence of glutaredoxin-like sequences in *Leishmania* is of interest as no typical glutaredoxins have been

previously described in any trypanosomatid. As described, *Leishmania* are likely to possess just one thioredoxin; this is probably the situation in other trypanosomatids. Thioredoxin is not essential in *T. brucei brucei* (Schmidt *et al.,* 2002) which has led to the authors' speculation that tryparedoxin, another small dithiol protein found in parasites with similar activities to thioredoxin, could be able to perform the cellular functions of thioredoxin. Certainly, degeneracy between glutaredoxins and thioredoxins is known to exist in other organisms (Potamitou *et al.,* 2002). The discovery of thioredoxin and putative glutaredoxins, as well as there being several proteins annotated as tryparedoxins, suggest degeneracy could also occur between these proteins in *Leishmania*. Indeed the similarities between glutaredoxins and oGSTs mean that degeneracy between these and TDR1 may occur in *Leishmania*; this may be important for analysis of TDR1 knock out parasites, as detailed in chapter five.

No canonical, cytosolic GSTs have been found in *Leishmania* by either biochemical methods or by searching the genome. This is perplexing as GSTs are found throughout nature in a wide range of organisms (for review see Hayes *et al.,* 2005). The recent discovery that elongation factor 1B from a variety of trypanosomatids display trypanothione S-transferase activity (Vickers and Fairlamb, 2004; Vickers *et al.,* 2004) may help explain the apparent absence of GSTs in *Leishmania*: trypanothione S-transferases could, in part, carry-out the functions usually performed by GSTs. However, given the range of GSTs found in most organisms, with their different sub-cellular localisations and subtrate specificities, it seems unlikely that just one protein could replace them. The absence of GSTs in *Leishmania* and the other trypanosomatids remains intriguing. Although oGSTs are functionally more similar to glutaredoxins than to other GSTs, they do exhibit low-level CDNB-conjugating activity typical of GSTs (Board *et al.,* 2000); TDR1 is therefore a candidate for functioning as a cellular GST.

The elucidation of the complete sequence of TDR1 revealed several interesting features. TDR1 has two halves that are similar to each other and it is therefore possible that the *TDR1* gene is a product of a smaller, ancestral gene that became duplicated. The differences between the two putative active sites of the protein are also intriguing as that in the C-terminal half contains just one cysteine (like GSTs and monothiol glutaredoxins) while the equivalent motif in the N-terminal half is has two cysteines (as glutaredoxins and thioredoxins do). Furthermore, these also differ from the predicted active site motifs from *T. cruzi* Tc52, the only known full-length homologue of TDR1 and may be suggestive of different functions. Although TDR1 was originally discovered because of its similarity to mammalian oGST which reduces methylated arsenicals, the biomethylation pathway is

probably not present in *Leishmania*. There are no reports of biomethylated species having been detected in parasites treated with antimonial compounds and no AS3MT homologue (the methyltransferase implicit in this pathway) was detected in the *L major* genome. However the recent observation that human oGST can reduce pentavalent inorganic arsenate as well as methylated forms (Aposhian and Aposhian, 2006) suggests that TDR1 could be capable of reducing antimonial drugs. The role of TDR1 as a metalloid reductase and in antimonial susceptibility will be investigated in the forthcoming chapters.

4 Analysis of TDR1: characterisation of recombinant protein and expression profile in *Leishmania*

It chapter three it was demonstrated that the *Leishmania major* genome contains an unusual gene, *TDR1*, which comprises two similar halves. When translated, both the 5' and 3' halves of TDR1 share sequence similarity with omega GSTs but also with other classes of GSTs, glutaredoxins and thioredoxins, as well as the only known full-length homologue, *Trypanosoma cruzi Tc52*. Although the sequences of translated TDR1 and Tc52 protein (which has been characterised to some extent (Moutiez *et al.*, 1995; Fernandez-Gomez *et al.*, 1998; Ouaissi *et al.*, 2002)) are considerably similar, it is notable that the predicted active site regions are not identical.

As discussed in chapter one, the effectiveness of pentavalent antimonials drugs used to treat leishmaniasis is likely to be dependent upon their reduction to a trivalent form (Shaked-Mishan *et al.*, 2001), a process which could be mediated by an arsenate reductase-like protein in *Leishmania*. However it seems likely that, with the exception of the recently identified *L. major* ACR2-like protein (Zhou *et al.*, 2004) and GAPDH, other known classes of arsenate reductases are not represented in the *Leishmania* genome. TDR1 was originally of interest due to its resemblance to omega GST (oGST), a recently discovered protein capable of reducing methylated arsenical compounds (Zakharyan *et al.*, 2001), and thus we hypothesised that it may be involved in the activation of pentavalent antimonials.

The work detailed in this chapter includes the production of recombinant TDR1 so that its *in vitro* functions, including whether it was able to reduce pentavalent metalloids, could be analysed. In addition the protein was assayed for its ability to mediate the oxidation of Sb(III). Furthermore, production of recombinant TDR1 would allow other interesting information to be gathered. There is no known quaternary structure data on Tc52, whereas human oGST forms a dimer (Board *et al.*, 2000) and glutaredoxins can be monomeric or dimeric (Kelley *et al.*, 1997; Noguera *et al.*, 2005). Other important considerations included the expression profile and sub-cellular localisation of TDR1. Pentavalent antimonials are toxic solely to the amastigote form of *Leishmania* (Ephros *et al.*, 1999); temporal variations in the level of a potential antimonial reductase could help explain this phenomenon. Insight into the position of TDR1 within the cell or the knowledge of whether it is released – as is proposed to be the case with Tc52 (Ouaissi *et al.*, 1995a; Ouaissi *et al.*, 1995b) – would also help establish a role for the protein.

4.1 Production of TDR1

4.1.1 Expression of TDR1 and 5' TDR1 in *E. coli*

Following the elucidation of the entire *TDR1* open reading frame by database mining and 3' RACE, the gene had been cloned into the pET28a(+) expression plasmid. In addition, the 5' half of the gene (*5'TDR1*) was cloned into the same vector. The 3' half of the gene (*3'TDR1*) had already been cloned into the expression vector by Dr. Helen Denton. The intention was to express the full-length TDR1 together with the 3' and 5' truncated proteins in order to analyse the individual halves of TDR1 and so clarify whether they had separate functions or if they were both required for a functioning protein Thereafter the plasmids were transformed into *E. coli* BL21(DE3) cells which were grown up to the appropriate density and treated with 2 mM IPTG to initiate protein expression. The cells were grown overnight at 15 °C before harvesting and lysing the cells, separating the soluble and insoluble cellular fractions in the process. SDS-PAGE analysis confirmed that the transformed cells were expressing proteins of 52 kD and 27 kD corresponding to the translated sizes of TDR1 and 5'TDR1, respectively. However, although approximately 50% of TDR1 being expressed was present in the soluble fraction, it appeared that all of the 5'TDR1 was insoluble (Figure 4.1). 3'TDR1 had been previously expressed and purified under similar conditions by Dr. Helen Denton and although it was partially soluble, the recombinant protein was highly unstable end therefore unsuitable for analysis.

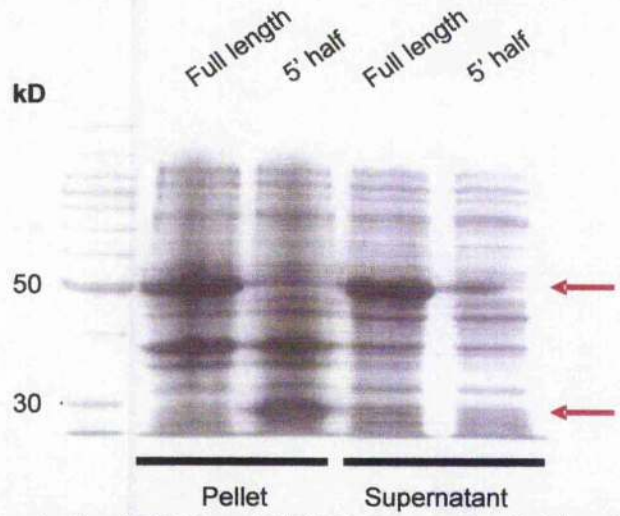

Figure 4.1: SDS-PAGE analysis of TDR1 and 5'TDR1 expression in *E. coli* soluble and insoluble fractions. Protein expression was induced and the cells were grown overnight at 15 °C. 1 ml of each was harvested and the insoluble and soluble fractions were separated by centrifugation following lysis by sonication. The insoluble pellet fractions were resuspended in a volume of 0.5 ml; 10 µl of each sample was mixed with 2 µl of 5 x protein sample buffer and loaded onto the 10% polyacrylamide gel for SDS-PAGE analysis. The gel was subsequently stained with coomassie blue. The 52 kD full length TDR1 is indicated with the uppermost red arrow and is present in both the soluble (supernatant) and insoluble (pellet) fractions while the 27 kD 5'TDR1 indicated with the lower red arrow is completely insoluble. In the left hand lane the Benchmark molecular weight marker is visible and sizes are indicated to the left in kD (kiloDaltons).

In order to obtain soluble 5'TDR1, a range of induction conditions and expression times were attempted by varying the IPTG concentration and bacteria growth period, respectively. The insoluble nature of the expressed protein may be due to inclusion bodies forming; a less vigorous production rate or a decrease in the amount of protein being made by the bacteria could prevent this. Unfortunately, none of the modified conditions yielded any soluble matter (Figure 4.2) and the decision was made to continue working solely on the full-length TDR1.

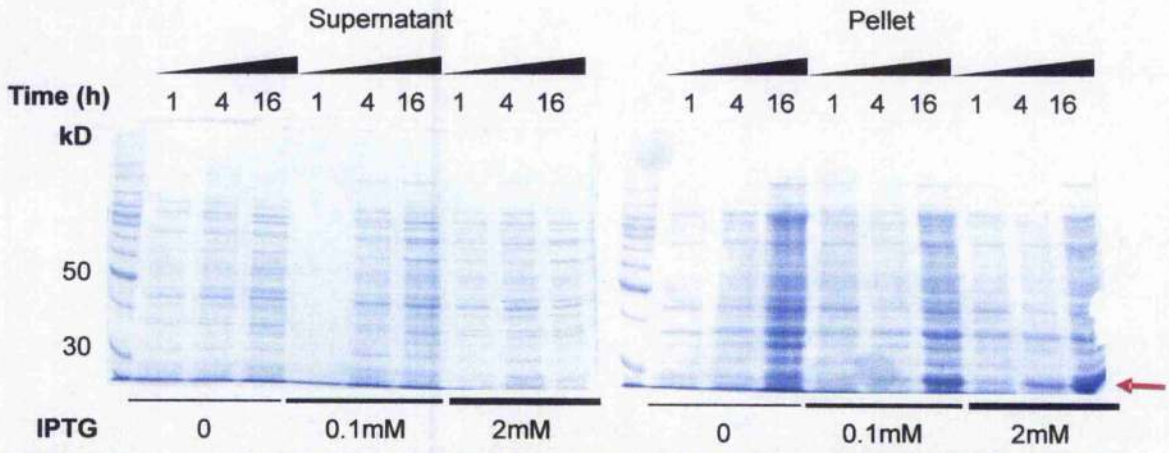

Figure 4.2: Varying expression conditions in an attempt to obtain soluble 5'TDR1. Protein expression was not induced or induced with 0.1 mM or 2 mM IPTG and the cells were grown at 15 °C for 1, 4 or 16 hours. 1 ml of each sample was harvested, resuspended in 250 µl and the insoluble and soluble fractions were separated by centrifugation following lysis by sonication. The insoluble pellet fractions were resuspended in a volume of 100 µl; 16 µl of each sample was mixed with 4 µl of 5 x protein sample buffer and loaded onto the 10% polyacrylamide gel for SDS-PAGE analysis. The gels were subsequently stained with Coomassie blue. Although more protein was produced with increased cell growth time and IPTG, the 27 kD 5'TDR1, indicated with the red arrow, is only present in the insoluble pellet fractions. In the left hand lane of each gel the Benchmark molecular weight marker is visible and sizes of the standards are indicated to the left of the figure in kD (kiloDaltons).

4.1.2 Purification of recombinant TDR1

TDR1 was purified from cellular lysates by Alan Scott and Dr. Helen Denton of University of Glasgow. The His-tagged recombinant protein was primarily purified from soluble *E. coli* lysates using a 13 ml Ni^{2+} -nitrilotriacetate affinity column and a BioCAD 700E workstation. Although only His-tagged proteins should be eluted, small quantities of contaminants can persist in the eluate from nickel-agarose columns and for this reason further purification was deemed necessary. *E. coli* glutaredoxin 2 is known to possess similar activities to oGST (Vlamis-Gardikas *et al.*, 1997); its presence in the purified TDR1 samples could obscure subsequent analysis. Following buffer exchange into 25 mM Tris-HCl, pH 7.9, the pooled TDR1-containing fractions were applied to a HQ column. At this pH, *E. coli* glutaredoxin 2 (pI 9.17), but not TDR1 (pI 6.3), was excluded from binding; TDR1 was eluted with a NaCl gradient and collected.

A yield of approximately 20 mg of protein was obtained from one litre of original bacterial culture. Recombinant TDR1 was found to be >95% pure as determined by SDS-PAGE and could be stored in elution buffer for periods of at least six months without deterioration. Unlike the truncated forms of TDR1 that were produced, full-length TDR1 was moderately soluble, stable and, as was later demonstrated, active (figure 4.3).

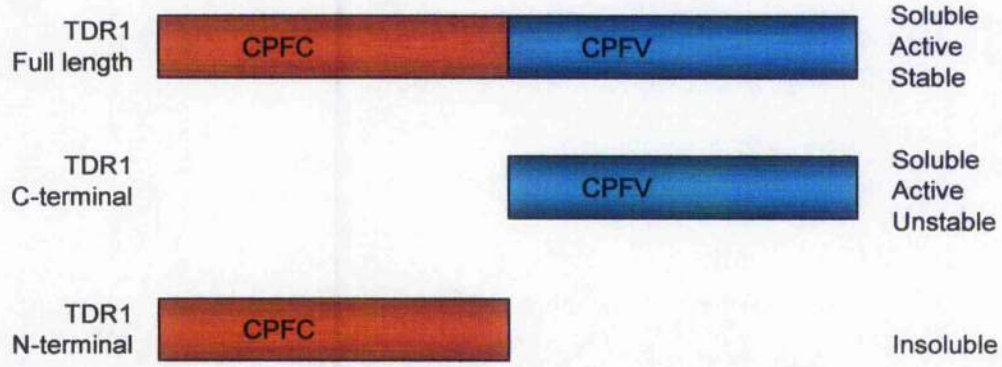

Figure 4.3: Schematic diagram representing the forms of recombinant protein produced and their properties. The predicted active site motifs are included on each similar half of the TDR1 protein. Only full-length recombinant TDR1 was soluble and stable.

4.1.3 Cleavage of the His-tag from recombinant TDR1

To facilitate purification, the pET28a(+) plasmid confers an N-terminal His-tag, comprising six histidine residues to the protein being expressed. Between the His-tag and the open reading frame lies a thrombin cleavage site, included so the His-tag can be removed from the recombinant protein after it has been successfully purified. The His-tag of recombinant TDR1 was cleaved off in this way (Figure 4.4). The absence of the His-tag results in a slight reduction in size of the protein, which corresponds to the loss of the 2 kD His-tag and can be visualised following SDS-PAGE. Successful cleavage was achieved after just two hours although subsequent reactions were performed for 18 hours as there was no protein degradation apparent and this incubation time would ensure complete His-tag removal. The loss of the His-tag did not affect the enzymatic characteristics of TDR1 and it was useful for ensuring that findings regarding the native molecular mass were due to interacting protein subunits rather than an effect of the tag.

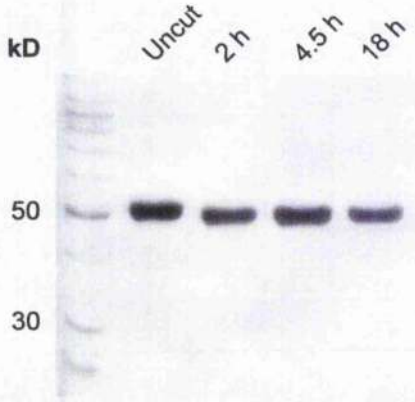

Figure 4.4: Cleavage of His-tag from recombinant TDR1. Thrombin cleavages were carried out at room temperature with 2 U of thrombin for every mg of recombinant TDR1. At different time-points (indicated above the lanes, h – hours) samples were taken to ensure there was no protein degradation and that successful cleavage (judged by slight band shift reflecting the loss of the His-tag). 2 µg of protein was loaded per lane onto a 12% acrylamide gel which was subsequently stained with Coomassie blue.

4.1.4 Isolation of TDR1 from *Leishmania* using S-hexyl-GSH Sepharose

In addition to producing recombinant TDR1 it was found that the native protein could be separated from *Leishmania* lysates with S-hexyl-GSH, using a similar technique to that employed in chapter three in an attempt to isolate *Leishmania* GSTs. A protein of 50 kD could be detected as a distinct band following SDS-PAGE analysis of the eluate; confirmation of its identity as TDR1 was achieved by western blotting (figure 4.5). This technique would prove useful as a means of effectively concentrating TDR1 from large volumes.

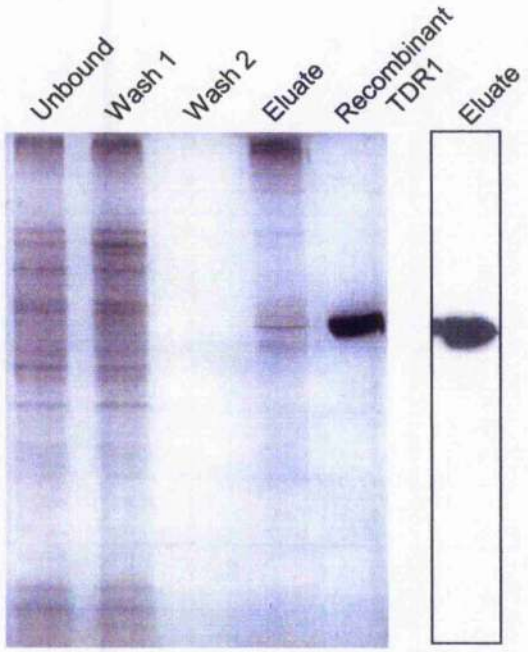

Figure 4.5: Isolation of TDR1 from *Leishmania major* using S-hexyl-GSH. In the first panel, fractions from a S-hexyl-GSH isolation of TDR1 from *L. major* (as indicated) were loaded onto a 10% acrylamide gel and subjected to SDS-PAGE analysis. The eluate contains a clear band similar in size to the 50 kD recombinant TDR1. The panel labelled 'western' is a western blot of the eluate sample present on the coomassie-stained gel using rabbit anti-TDR1, confirming the identity of the 50 kD band as TDR1

4.2 Biochemical characterisation of recombinant TDR1

Recombinant protein produced, purified and stored as described above was used for all investigations into the catalytic properties of TDR1 of *L. major*. For studies on the native molecular mass of TDR1, protein that had had the His-tag removed was also used. The biochemical characterisation of recombinant TDR1 is presented and discussed in Denton *et al.*, 2004 and was carried out by Dr. Helen Denton of Glasgow University; accordingly it has not been summarised here.

4.3 Crystalisation of recombinant TDR1

In collaboration with Professor Bill Hunter and Dr. Karen McLuskey (both University of Dundee), TDR1 crystalisation trials were attempted. This was in an effort to obtain TDR1 crystals suitable for X-ray diffraction, so that structural information could be gathered. The trials were set up with TDR1 with and without GSH as well as with and without the His-tag but crystals were obtained with the His-tagged form only. The crystals formed were highly reproducible and measured approximately 0.15 mm x 0.15 mm x 0.05 mm in the absence of GSH, and 0.15 mm x 0.15 mm x 0.15 mm with GSH. Unfortunately, both sizes of crystals were not of particularly good quality, diffracting to just 8 Å. In order for structural information to be obtained, the crystals have to diffract to 3 Å and so this approach did not yield any information about regarding the native configuration of TDR1.

4.4 Temporal Expression of TDR1

Characterisation of when in the parasite life cycle TDR1 was expressed would help clarify whether the enzyme contributed to the amastigote-specific toxicity of pentavalent antimonials. TDR1 expression was analysed in several *Leishmania* species by western blotting using purified anti-TDR1 anti-serum from rabbit and anti-TDR1 anti serum from sheep. Both sera had been shown to recognise recombinant TDR1 on dot-blots.

4.4.1 *L. mexicana* Axenic Amastigote-like Forms Display a Similar Protease Expression Profile as Lesion Amastigotes

L. mexicana can be grown axenically as amastigote-like forms as well as promastigotes (Bates *et al.*, 1992). However, before using them in experiments it was considered necessary to confirm the status of the forms as amastigotes by a means other than microscopic analysis. The protease expression between *L. mexicana* amastigotes and

promastigotes differs substantially and is therefore a good indicator of life-cycle stage (Bates *et al.*, 1992). Using gelatine SDS-PAGE, the protease activities of the putative *L. mexicana* axenic amastigotes were compared to those of *L. mexicana* promastigotes and amastigotes purified from animals (figure 4.6). It is clear that the protease activities of the axenic amastigotes resemble those of the animal amastigotes much more closely than those of the promastigotes. Together with the amastigote-like morphology of these cells, this result indicates these axenic parasites can be viewed as amastigotes with some degree of confidence.

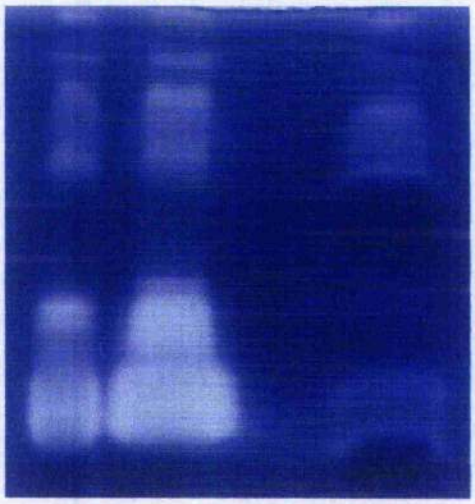

Figure 4.6: Gelatin gel showing protease activities in different *L. mexicana* life-cycle stages. Axenic amastigotes were grown in SDM pH 5.5 at 32 °C. Protein samples were separated on a 12% acrylamide gel containing 0.2% gelatin by SDS-PAGE. The gel was incubated at 37°C for 1.5 hours in sodium acetate pH 5.5 before staining with Coomassie blue. Lane 1, 12 µg axenic amastigote total protein; lane 2, 12 µg lesion amastigote total protein; lane 3, 60 µg promastigote total protein.

4.4.2 Detection of TDR1 in the Soluble Fraction of *Leishmania*

L. major, *L. mexicana* and *L. infantum* promastigotes harvested in the stationary growth phase were all examined for TDR1 expression. As described in chapter two, harvested parasites were separated into soluble and insoluble fractions following lysis by sonication. Western blotting revealed that the sheep anti-serum recognised a protein of approximately 50 kD in *L. major and L. mexicana* (Figures 4.7A and 4.7B), which corresponds to the predicted size of TDR1. The protein was slightly smaller than recombinant TDR1, a difference in size that was attributed to the His-tag being absent in the native form of the

protein, and was present in the soluble fractions only. Similar amounts of TDR1 were present in *L. mexicana* promastigotes and axenic amastigotes.

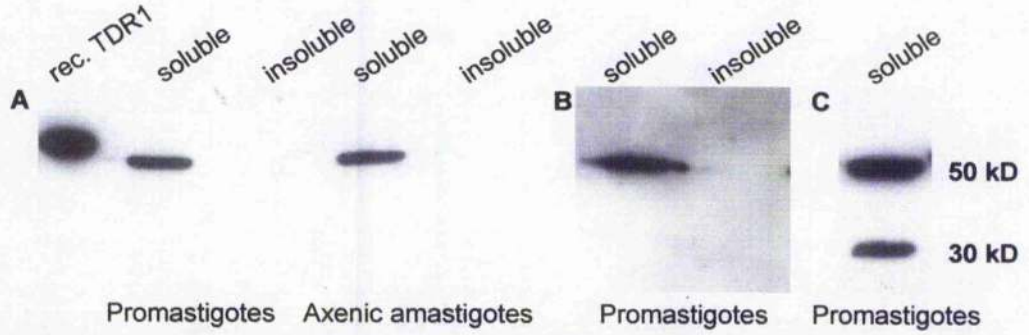

Figure 4.7: TDR1 expression in *Leishmania*. Western blot analysis of TDR1 expression in different *Leishmania* lysates following protein separation by SDS-PAGE. In each case at 10-20 μg of total protein was loaded per lane. (**A**) *L. mexicana* soluble and insoluble extracts of both promastigotes and axenic amastigotes alongside 100 ng of recombinant TDR1 protein. (**B**) *L. major* soluble and insoluble promastigote extracts. (**C**) *L. infantum* soluble extract.

The anti-sera also recognised a protein of a similar size in *L. infantum* lysates (Figure 4.7C). Following SDS-PAGE, the *L. infantum* protein ran slightly smaller than that of *L. major*: this could be due to subtle differences between the protein amino acid sequences. The sheep anti-sera also recognised a protein of approximately 30 kD in the *L. infantum* lysates that was absent on the western blots from the other *Leishmania* species. This unknown protein is probably recognised due to it having a similar epitope to either TDR1 or a protein present in trace amounts in the original recombinant TDR1 antigen sample. The possibility of it being a truncated or processed version of TDR1 has also been considered.

4.4.3 TDR1 is uniformly expressed in *L. major* promastigotes

Although the *L. major* life-cycle has primarily two distinct stages, promastigote and amastigote, several different naturally occurring developmental forms of promastigotes are recognised that could potentially exhibit varying levels of protein expression. These can be broadly divided into early log (when the parasites are dividing but remain at a relatively low concentration of up to ~4 x 10^6 cells/ml), late log (when the parasites are dividing but are becoming more concentrated in the media at up to ~2 x 10^7 cells/ml) and stationary phase (the parasites have ceased to divide and remain concentrated; metacyclic parasites appear). Western blotting of *L. major* lysates obtained during these different promastigote

life cycle stages revealed that TDR1 expression appeared to remain at a similar level (Figures 4.8A). There was either no fluctuation in expression in promastigotes, or any changes were too minor to detect using this method.

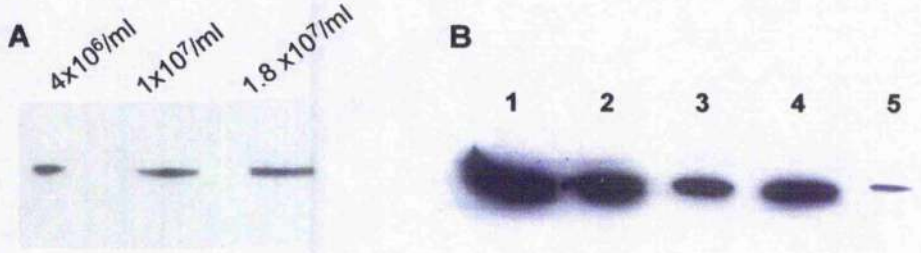

Figure 4.8: Western blot analysis of TDR1 expression in different *L major* life-cycle stages. (**A**) Different growth phases of *L. major* promastigotes. The densities of the parasites when they were harvested are indicated and after lysis and isolation of the soluble fraction approximately 10 µg of total protein was loaded per lane. (**B**) Comparison of TDR1 expression between *L. major* promastigotes and amastigotes. Lane 1, 200 ng of recombinant TDR1; lane 2, 100 ng of recombinant TDR1; lane 3, 50 ng of recombinant TDR1; lane 4, S-hexyl-GSH binding fraction recovered from 0.23 mg of protein from the amastigote lysate; lane 5, S-hexyl-GSH binding fraction recovered from 0.23 mg of protein from the promastigote lysate.

4.4.4 Increased expression of TDR1 in *L. major* amastigotes

As stated, only *Leishmania* amastigotes are susceptible to pentavalent antimonials and this phenomenon has been attributed to a life cycle-specific ability to reduce the drug to the more toxic trivalent form (Shaked-Mishan *et al.*, 2001). *L. major* amastigotes were extracted and purified from animals and so TDR1 expression could be compared between these and promastigotes grown in culture. The lysates were equalised for initial protein concentration before incubation with S-hexyl-GSH-agarose so the TDR1 present in each sample could be isolated. The eluate from each was subjected to SDS-PAGE and western blotting using the purified rabbit anti-serum revealed bands corresponding to the molecular mass of TDR1 (Figure 4.8B), although interestingly it was apparent that more TDR1 was present in the amastigote sample. It was estimated that approximately ten times more TDR1 was recovered from the amastigote lysate than from the equivalent promastigote lysate.

4.5 Spatial Expression of TDR1

Analysis of whether TDR1 is secreted and the cellular distribution of the protein were of interest both to help identify a possible site of antimonial accumulation and reduction, and to further clarify the endogenous role of TDR1. In addition, it was of interest to ascertain whether the location of TDR1 in the cell was similar to that of Tc52 or oGST, although investigation into the spatial expression of the latter has mainly focused on tissue-level rather than sub-cellular localisation. The sheep anti-TDR1 serum was used for these investigations

4.5.1 Analysis of Secretion of TDR1 from *L. major*

Investigations into whether a protein is released from a cell can be problematic due to the difficulty in detecting a given protein when it is dilute in large volumes. Here the fact that TDR1 binds to S-hexyl-GSH was exploited so that spent culture media could be analysed for the presence of the protein. Western blot analysis showed that TDR1 could be detected in samples eluted from the S-hexyl-GSH, when it had been initially incubated with 10 ml of filtered, spent, *L. major* promastigote culture media (Figure 4.9). As a control, 10 ml of fresh culture media was 'spiked' with 160 ng of recombinant TDR1, and subjected to the same incubation with S-hexyl-GSH and elution conditions as the other sample. The amount of TDR1 used in the control was approximately the total amount of TDR1 present in 2×10^8 *L. major* promastigotes harvested while in the stationary phase of growth, as determined by previous western blot experiments. Several times more recombinant TDR1 was detected in the control sample than from the spent media, suggesting that the amount of TDR1 secreted by the cells is considerably less than that retained in the parasite. However, this may not be the case as secreted protein may be subjected to proteolysis or bound to other proteins, therefore hampering the recovery of the protein. The finding that TDR1 is secreted could implicate the protein as being potentially involved in host-parasite interactions. As mentioned, Tc52 is also thought to be released from cells (Schoneck *et al.*, 1994) and has been attributed such a role.

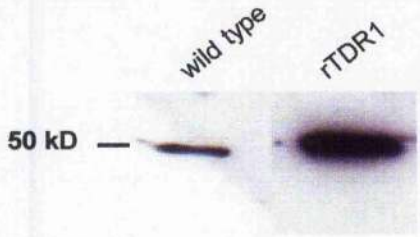

Figure 4.9: Western blot analysis of secretion of TDR1 from *L. major*. Seven-day old spent media and fresh, pH-adjusted media that had been 'spiked' with 160 ng of recombinant TDR1 (necessary as a positive control) were filtered before incubating with S-hexyl-GSH (as previously described). The eluted fractions were subjected to SDS-PAGE and western blot analysis using sheep anti-TDR1. A 50 kD was detected in both the *L. major* spent media sample (labelled wild type) and the TDR1-spiked positive control sample (labelled rTDR1).

4.5.2 Immuno-localisation of TDR1 in *L. major* Promastigotes

Immuno-localisation studies were performed on wild-type *L. major* promastigotes, as well as on a TDR1 KO *L. major* line that had been generated (see chapter five). The staining was not solely associated with the cell nucleus or kinetoplast but was dispersed throughout the cell (Figure 4.10A). In some images there seemed to be some concentration of signal occurring, particularly around the kinetoplast and between the nucleus and kinetoplast, suggesting that the enzyme may be to some extent located in an as yet unidentified organelle. However, the fluorescence was observed throughout the parasites and was quite diffuse which is more indicative of TDR1 being cytosolic. The vast reduction in intensity of a signal from the TDR1 KO line under the same experimental conditions provided evidence that fluorescence visible in the wild-type line was largely TDR1-specific (Figure 4.10B). Previous reports have shown that Tc52 is localised in reservomeres (Ouaissi *et al.*, 1995b) but is also deemed to be a secreted protein (Ouaissi *et al.*, 1995a). Meanwhile human oGST2 has been found to be both cytosolic and nuclear (Wang *et al.*, 2005).

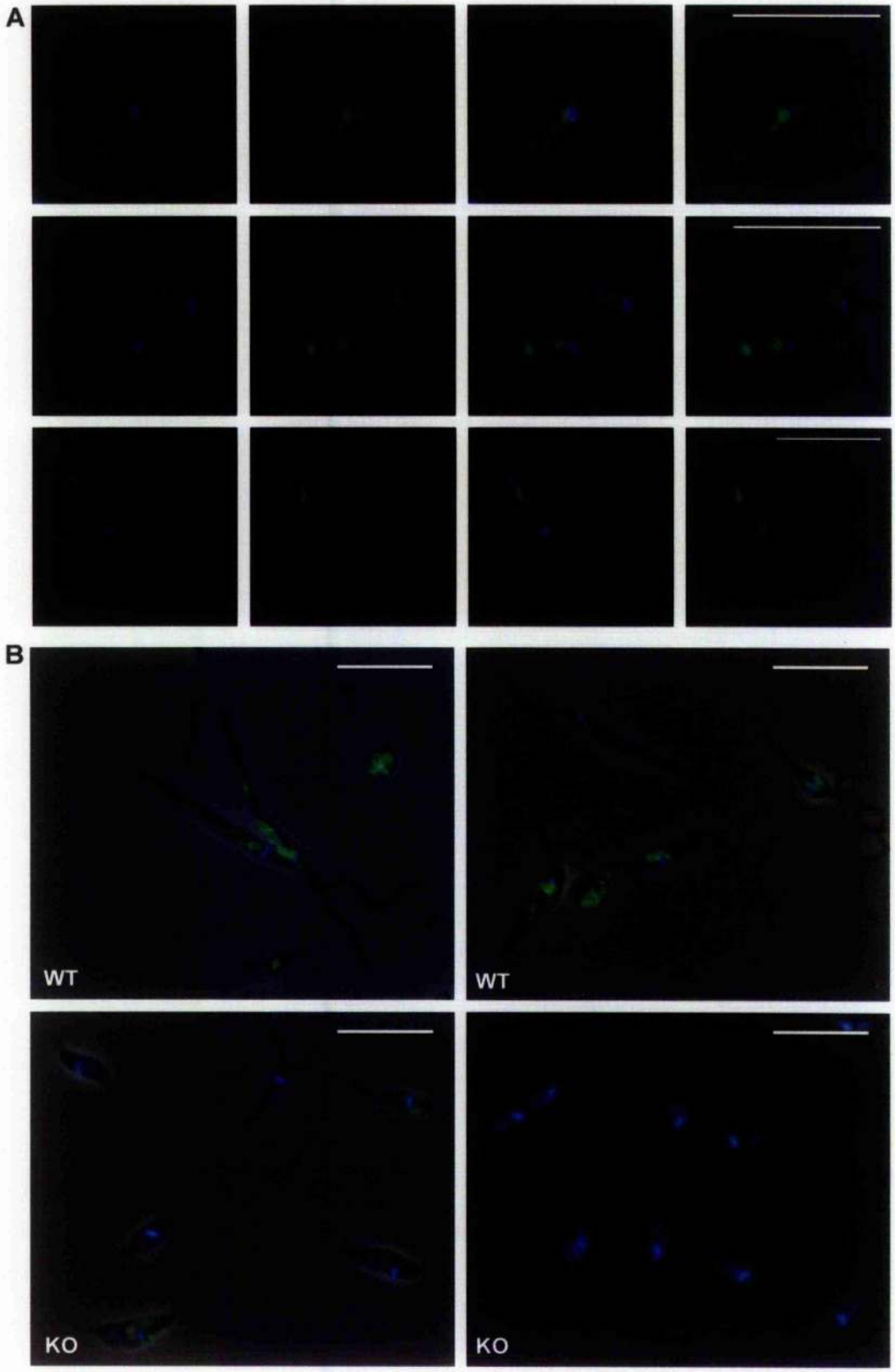

Figure 4.10: Immunolocalisation of TDR1 in *L. major* promastigotes. (**A**) Distribution of TDR1 within wild-type promastigotes. The blue staining shows DAPI, highlighting the nucleus and kinetoplast; the green stain shows the secondary antibody, Alexa-Fluor 488, and corresponds to TDR1 distribution. Merged images are shown alone and within the context of the DIC parasite image. (**B**) Comparison of TDR1 immunolocalisation signals between wild type (WT) and TDR1 knock-out (KO) *L. major* parasite lines. The Alexa-Fluor 488 secondary antibody signals (green staining) are comparable between panels, as experimental and image-processing conditions were equivalent. In all images the primary antibody (sheep anti-TDR1) was applied at a dilution of 1/100 and the secondary antibody (as above) was applied at a dilution of 1/500. The white scale bar shown on each image represents a distance of 10 μM.

4.6 Analysis of oxidation of trivalent antimonials by hydrogen peroxide

Although it is well established that pentavalent arsenicals and antimonials can be reduced, both enzymatically and directly upon reaction with thiols, little is known about whether the reverse reaction also persists. If oxidation of trivalent metalloids was possible in cells this could alter the perception of antimonial sensitivity: an increase in trivalent species oxidation rather than a decrease in pentavalent species reduction could result in drug resistance. Recently it was reported that trivalent arsenite could be oxidised to pentavalent arsenate by hydrogen peroxide (H_2O_2) (Aposhian *et al.*, 2003). This was the first published account that alluded to the potential importance of this mechanism in the detoxification of trivalent metalloids in a biological system. However, there are accounts of bacteria and archaea that are capable of oxidising arsenite (and indeed antimonite (Lialikova, 1974)) and are deemed to possess arsenite oxidase enzyme activity. Several proteins have been implicated in oxidising arsenite and the best characterised are the aoxA and aoxB proteins originally found in *Alcaligenes faecalis* (Anderson *et al.*, 1992). However, the *L. major* genome does not contain sequences with significant homology to either of these genes and accordingly, investigations into whether metalloid oxidation is possible in *Leishmania* have focused on the role of H_2O_2. As described, the BPR assay was used to detect trivalent antimonial species present. For each experiment, a standard curve of potassium antimonial tartrate (KAT) versus the absorbance at 540 nm was constructed, and the amount of trivalent KAT remaining at each time point was calculated from that.

4.6.1 Trivalent potassium antimonyl tartrate is oxidised by H_2O_2

In the presence of H_2O_2, the amount of trivalent antimony present in the sample decreased over time (Figure 4.11). The elimination of the trivalent species was dependant on the amount of H_2O_2 present in the reaction and when there were equal concentrations of KAT and H_2O_2 present >90% of the KAT was removed after 130 minutes. However, the molar relationship between H_2O_2 present and KAT oxidised does not appear to be 1:1. For example, when the concentration of KAT is 1 mM and H_2O_2 is 0.5 mM, the percentage of KAT oxidised is ~70%. The elimination of KAT was rapid and when higher concentrations of H_2O_2 were present much of the KAT had already been oxidised when the first time point was taken. The BPR assay can only be used to determine trivalent antimonials species present and so the identity of the product is unknown. However the analogous reaction with trivalent arsenite yields a pentavalent product (Aposhian *et al.*, 2003).

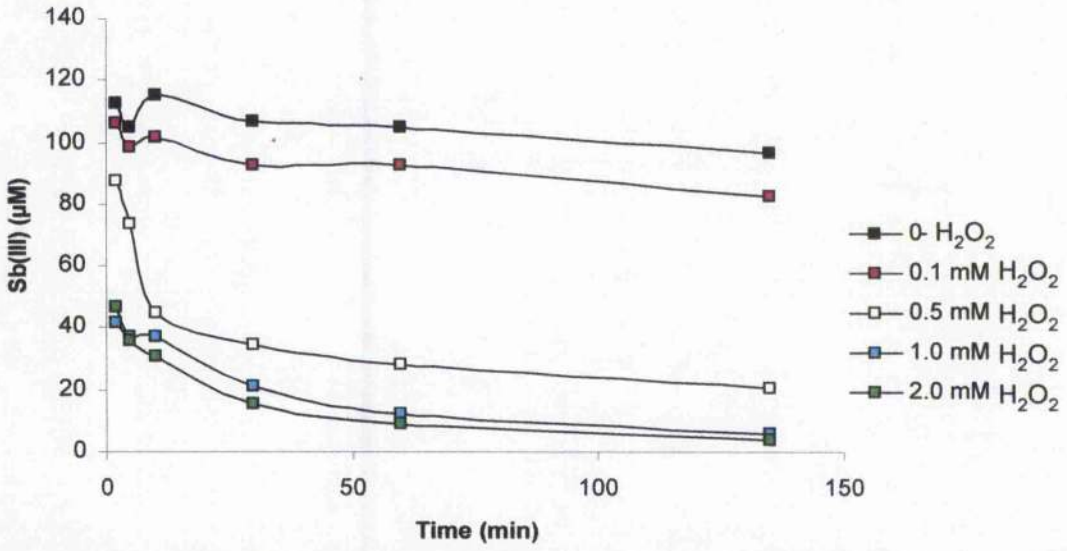

Figure 4.11: Trivalent KAT is depleted by H_2O_2. 1 mM KAT was incubated at room temperature with 0, 0.1 mM, 0.5 mM, 1 mM or 2 mM H_2O_2 in 0.1 M Tris pH 8.0 in a final volume of 1 ml. Sb(III) depletion was followed discontinuously by the BPR assay in which the samples were diluted 1/10. Points represent an average of two experiments.

4.6.2 The effect of pH and recombinant TDR1 on oxidation of KAT by H_2O_2

In order to determine the optimal conditions for the oxidation of KAT by H_2O_2 the reaction was set up at a range of pH values. It was found that the oxidation did not occur when the pH was below 7.0 and as the pH was raised the reaction rate increased. It is worth noting that a basic environment was also required for the oxidation of trivalent antimonials by hydrogen peroxide in other studies (Quentel *et al.*, 2004). In order to keep the experiments as physiologically relevant as possible, all subsequent investigations were carried out at pH 8.0.

It has been shown previously that some glutaredoxins can act as peroxidases in the cell by detoxifying H_2O_2 (Collinson *et al.*, 2002). Due to the sequence similarities that exist between these proteins and TDR1, recombinant TDR1 was added to the reactions at this stage so any affect its presence caused could be observed. If TDR1 could detoxify H_2O_2 then this may prevent any oxidation of trivalent antimony while it was also possible that TDR1 could act as an oxidase and catalyse the antimonial oxidation. The effect of adding GSH and GSSG as well as TDR1 was also monitored (see below). However, the addition of recombinant TDR1 neither catalysed nor inhibited the oxidation of KAT (Figure 4.12); this was also the case at lower pH values.

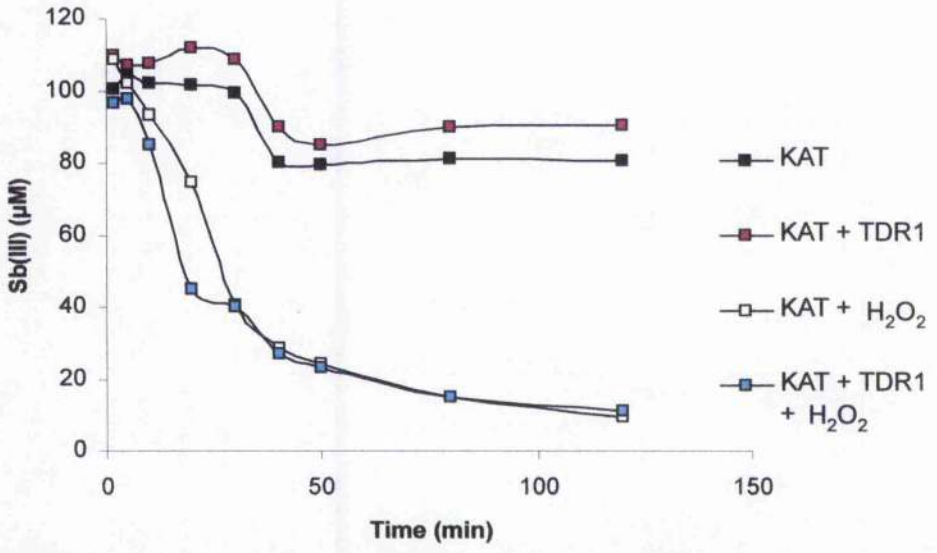

Figure 4.12: Recombinant TDR1 has no effect on the oxidation of KAT by H_2O_2. 1 mM KAT was incubated at room temperature with 1 mM H_2O_2, 4 μg TDR1 or both, in 0.1 M Tris pH 8.0 in a final volume of 1 ml. Sb(III) depletion was followed discontinuously by the BPR assay in which the samples were diluted 1/10. Points represent an average of two experiments.

4.6.3 The trivalent product formed upon reaction of TDR1 with sodium stibogluconate is oxidised by H_2O_2

KAT is a trivalent antimonial compound that is not used as an antileishmanial drug and therefore it was desirable to establish whether H_2O_2 could oxidise additional trivalent antimonials that may be more relevant. As described, the product of the reaction between sodium stibogluconate and TDR1 is a trivalent species and is likely to actually be encountered by the parasite. Therefore the product of TDR1 activity on stibogluconate was quantified (using the BPR assay) and added to the reaction rather than KAT. H_2O_2 was also found to be capable of oxidising this trivalent antimonial (Figure 4.13), indeed the rate of oxidation was faster than that achieved when KAT was the trivalent substrate. Again, without more in depth analysis it is impossible to be certain of the identity of the species formed but it is not unreasonable to speculate that it may have converted back to sodium stibogluconate. This is the first evidence that the parasitic metabolism of antimonial drugs does not necessarily consist of straightforward reduction from a pentavalent to a trivalent form. If it is possible for more toxic, trivalent antimonials, formed by reduction of the pentavalent drugs administered, to be converted back to the less toxic pentavalent form this has clear implications for the anti-parasitic activity of the drugs. Intracellular H_2O_2 concentration could be a factor in antimonial toxicity, as is discussed in section 4.7.

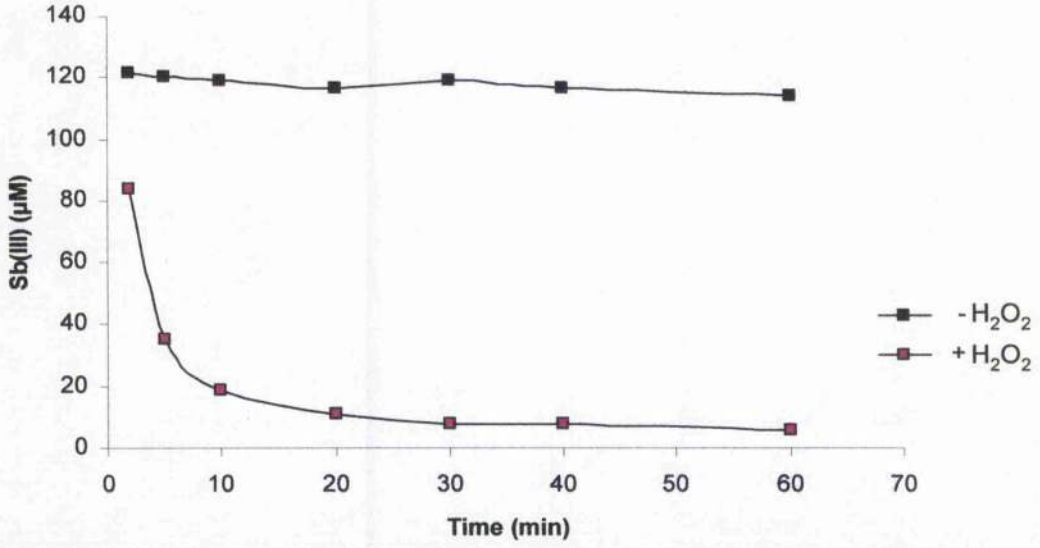

Figure 4.13: The trivalent antimonial formed upon reaction of TDR1 and sodium stibogluconate is oxidised by H_2O_2. Sodium stibogluconate was incubated overnight with TDR1 and GSH and the trivalent product was quantified using the BPR assay. 1 mM of the product was incubated at room temperature with or without 1 mM H_2O_2 in 0.1 M Tris pH 9.0 in a final volume of 340 µl. The resulting pH of the assay was pH ~8.0. Sb(III) depletion was followed discontinuously by the BPR assay in which the samples were diluted 1/10. Points represent an average of two experiments.

4.6.4 GSH Inhibits Oxidation of KAT by H_2O_2 while GSSG has no effect on the reaction

GSH is known to behave as a cellular anti-oxidant and react with H_2O_2 to form $2H_2O$. Therefore its presence may prevent the oxidation of trivalent antimonials from proceeding, especially when it exists at a high level as reported by Ariyanayagam and Fairlamb 2001. GSH was added to the reactions and was found to decrease the amount of KAT oxidised (Figure 4.14). This is probably due to H_2O_2 reacting with the two molecules of GSH to form GSSG and H_2O and therefore depleting the amount of H_2O_2 available to react with the antimonial. Curiously, the initial speed of the reaction appeared to be increased in the presence of GSH as in the first time point there is significantly less KAT remaining in the sample with GSH added than in that without (figure 4.14), although the overall level of oxidation was reduced. Due to the high intra-cellular concentrations of GSH, this result has significant implications for whether the feasibility of cellular oxidation of trivalent antimonials.

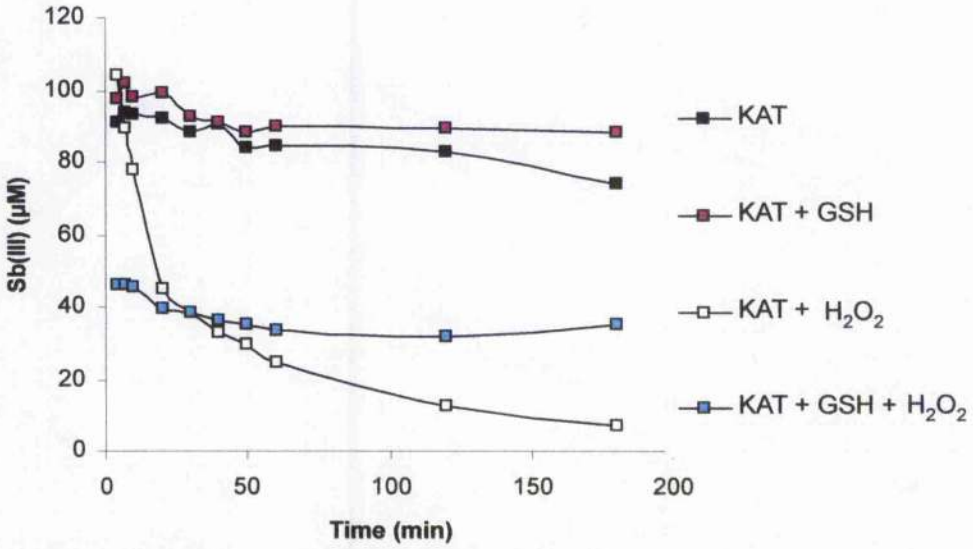

Figure 4.14: GSH reduces the amount of oxidation of KAT by H_2O_2. 1 mM KAT was incubated at room temperature with 1 mM H_2O_2, 1 mM GSH or both, in 0.1 M Tris pH 8.0 in a final volume of 1 ml. Sb(III) depletion was followed discontinuously by the BPR assay in which the samples were diluted 1/10. Points represent an average of two experiments.

Due to the ability of the reduced from of glutathione – GSH – being capable of directly reducing pentavalent antimonials, the possibility that the oxidised form – GSSG – could be involved in oxidising trivalent antimonials was considered. It was also possible that GSSG could affect the oxidation by H_2O_2. However, GSSG appeared to have no effect on the trivalent nature of KAT, or on the oxidation reaction (figure 4.15). However the rapid oxidation of KAT by H_2O_2 resulted in much of the KAT being eliminated before the initial rime point was taken, so any effect by GSSG on the reaction during this period cannot be monitored. As mentioned, the presence of TDR1 in these reactions resulted in no significant further effect.

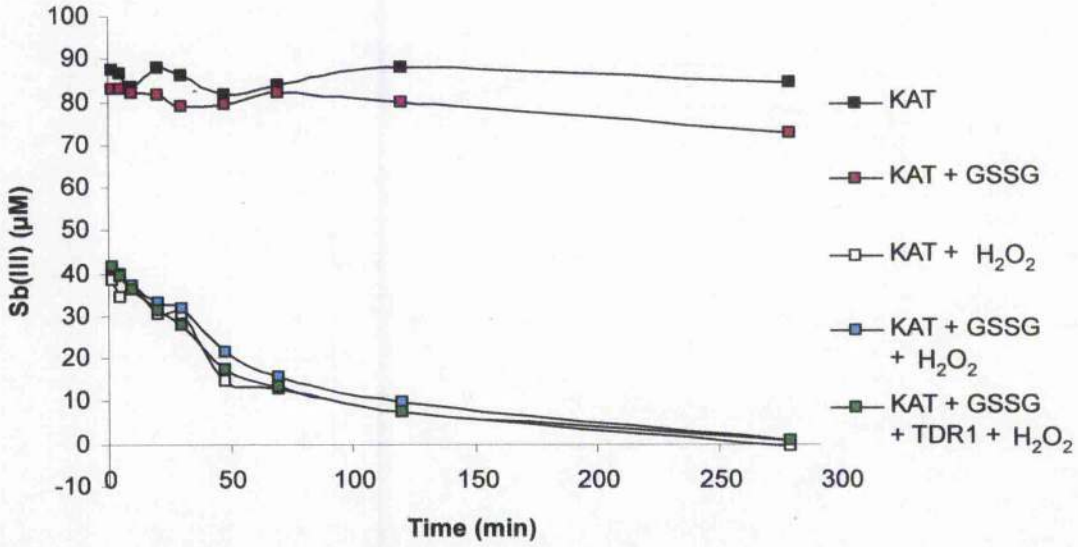

Figure 4.15: GSSG has no effect on the oxidation of KAT by H_2O_2. 1 mM KAT was incubated at room temperature with 1 mM H_2O_2, 1 mM GSSG or both, with or without 3 μg TDR1. The reaction was in 0.1 M Tris pH 8.0 in a final volume of 1 ml. Sb(III) depletion was followed discontinuously by the BPR assay in which the samples were diluted 1/10. Points represent an average of two experiments.

4.6.5 Analysis of KAT and Sodium Stibogluconate upon Incubation with *Leishmania*

It was decided to employ the BPR assay to monitor whether any changes in the state antimonials could be detected upon incubation with *Leishmania*. *L. mexicana* amastigote and *L. major* promastigote cultures were grown as standard, except that either KAT or sodium stibogluconate (or extra media as a negative control) were added to a final concentration of 200 μM. Every day for 10 days a sample was removed from each culture, the parasites were removed by centrifugation, and 50 μl of the spent media was subjected to analysis in a final volume of 200 μl using the BPR assay. The assay only detects trivalent species and so any decrease in trivalent antimonial level detected could be interpreted as either oxidation or as uptake by the parasites. However there was no detected decrease in the level of trivalent antimonial species when KAT was administered or formation of trivalent species when sodium stibogluconate was administered, when incubated with amastigotes or promastigotes. However, it is likely that the assay is too insensitive to detect any changes in the state of the drugs or uptake that was occurring.

4.7 Discussion

L. major TDR1 was originally discovered – and was of interest – due to its similarity to omega GSTs, which was suggestive that the protein could be involved in reducing antileishmanial antimonials and therefore mediating drug-susceptibility. Upon analysis of the TDR1 ORF it became clear that TDR1 encoded a highly unusual protein containing two omega GST-like domains with different predicted active sites. Together with the observation that no other GSTs have been identified in *Leishmania*, these factors indicate the potentially fascinating activities of TDR1. Characterisation of recombinant TDR1 and analysis of its cellular expression profile were intended to help elucidate which of the proteins many inferred functions were accurate.

Due to the nature of the two-domain structure of TDR1, it was desirable to obtain separate C- and N- terminal half-length proteins, as well as the full-length recombinant protein. It was hoped that this approach would provide an insight into whether the protein functioned as a whole or if each half was capable of acting independently. However, despite varying the expression-inducing IPTG concentrations and growth times of the *E. coli* expression cells, acquiring soluble and stable versions of these truncated proteins was problematic. At this point it was considered whether further attempts could have been made to obtain the half-length proteins. For example, re-cloning the gene fragments in expression vectors that add differently placed expression tags or alternative types of tag; or expressing the protein in a eukaryotic system. However, it was decided that these approaches could have been time-consuming and were without guarantee of a successful outcome; therefore they were discounted.

Meanwhile, satisfactory levels of active full-length TDR1 were produced quite easily with approximately 20 mg of protein being recovered from one litre of bacterial culture. This relatively high yield was achieved despite the additional purification steps taken to eliminate any potentially contaminating proteins from the sample, specifically *E. coli* glutaredoxin 2. It is interesting to note that there is no published account of enzymatically active recombinant Tc52 - the most similar known protein to TDR1 - being produced. In all reports native protein was characterised after purification from *T. cruzi* lysates with S-hexyl-GSH (Moutiez *et al.*, 1995) suggesting that production of recombinant Tc52 is not straightforward. Analysis of the recombinant protein revealed several enzymatic capabilities of TDR1, summarised in Denton *et al.*, 2004.

All the species analysed by western blot – *L. major*, *L. mexicana* and *L. infantum* – expressed TDR1 and the protein was detected in all life-cycle stages of *L. major*. This apparently ubiquitous expression, together with the highly conserved amino acid sequence between the proteins, could indicate that TDR1 has a vital role in the parasite. Indeed Tc52 has been reported to be essential (Allaoui *et al.*, 1999) with the authors being unable to obtain Tc52 knock out *T. cruzi* strains. However, as will be shown in chapter five this is not the case with TDR1, providing further evidence that TDR1 and Tc52 have divergent roles.

Another area in which the two proteins differ is in their cellular localisation. Within *T. cruzi* epimastigotes, Tc52 is reported to reside in vesicles resembling the reservomere (Ouaissi *et al.*, 1995b), large acidic organelles found towards the posterior end of the parasite. This contrasts with the distribution of TDR1 in promastigotes, which was quite diffuse and may be cytosolic, and certainly was more concentrated at the anterior end of the parasite. However, the authors did not perform any co-localisation studies, using antibodies to characterised marker proteins to directly compare the distribution signals, and therefore the inferred localisation of Tc52 is not certain. This is also the case with the TDR1 localisation study, although the comparative lack of staining detected in the TDR1 knock out line at least indicates that the distribution pattern is specific to TDR1. Analysis of TDR1 localisation in amastigotes – either axenic or isolated from animals – would also have been desirable as the protein may behave differently between life-cycle stages. For example the protein may be secreted from metacyclics and amastigotes but not procyclics. TDR1 was detected by western blot in spent media from a stationary phase *L. major* promastigote culture. Although the media was filtered prior to analysis, the possibility that dead parasites may release TDR1 upon degradation, rather than it being actively secreted, cannot be ruled out. Analysis of secretion from *L mexicana* axenic amastigotes – spent media from *L. major* lesion amastigotes would be difficult to obtain – would be potentially interesting. Tc52 is reportedly secreted (Schoneck *et al.*, 1994) from epimastigotes, having an important role in aiding the parasite to modulate the mammalian host's immune response to infection (Garzon *et al.*, 2003). Whether TDR1 has a similar role in *Leishmania* has not been investigated as part of this study, though if it is secreted by intracellular stages of the parasite this could help explain it's involvement in parasite infectivity, discussed in chapter five.

The temporal expression pattern of TDR1 is another area that potentially differs from that of Tc52. TDR1 expression is uniform throughout the various growth phases of *L. major* promastigotes while an increase in expression was detected in lesion amastigotes relative

to the promastigotes. In *T. cruzi*, Tc52 is most highly expressed in the epimastigote and amastigote stages, and is less so in trypomastigotes (Ouaissi *et al.*, 1995b). In *L. mexicana* axenic amastigotes the same increase in TDR1 expression was not observed: this could be due to the axenic nature of the culture not entirely reflecting the natural environment encountered by amastigotes and therefore affecting gene expression. However the lesion amastigote-like protease expression profile of the axenic amastigotes suggested otherwise. Equally, the differences in TDR1 amastigote expression levels may be due to variations between species. The implications of this apparent up-regulation may be significant.It is generally accepted that pentavalent antimonial drugs are toxic specifically to amastigotes yet it is not known if this is due to increased parasite susceptibility or stage-specific reduction – and hence activation – of the pentavalent compounds. Controversy also persists over whether activation occurs in the macrophage or the parasite (Ephros *et al.*, 1999, Sereno *et al.*, 1998). It has been reported that only amastigotes and not promastigotes are capable of reducing pentavalent antimonials and that Pentostam-resistant *L. donovani* parasites were deficient in this activity (Shaked-Mishan *et al.*, 2001). Although it has been demonstrated that some thiols are capable of directly reducing pentavalent antimonials (Frezard *et al.*, 2001), the rate is very low. In chapter five, investigations on *L. major* lacking or expressing an increased amount of TDR1 are presented, which help clarify whether TDR1 is involved in mediating toxicity of pentavalent antimonial drugs.

Finally, the revelation that H_2O_2 can oxidise KAT and even more intriguingly, the trivalent product formed upon reaction between TDR1 and sodium stibogluconate, shows that the metabolism of pentavalent antimonials may not be as straightforward as once thought. It is clearly possible for trivalent antimonials. Therefore reduction of the pentavalent drug administered could occur, but whether the product remains in the more toxic trivalent form or is oxidised back to a pentavalent species may be dependant upon the concentration of H_2O_2 present. Indeed it is possible that other reactive oxygen species found in *Leishmania* could also be capable of causing any trivalent antimonial species formed to be oxidised. However, the observation that GSH inhibits the oxidation of KAT brings in to question the physiological relevance of the oxidation of trivalent antimonials. The concentration of GSH in *L. donovani* promastigotes is approximately 4 mM while $T(SH)_2$, which also reacts with H_2O_2 and is therefore likely to inhibit KAT oxidation in a similar way, is approximately 5.2 mM (Ariyanayagam and Fairlamb 2001). Although no accounts of H_2O_2 concentration in *Leishmania* could be found, in *T. brucei* H_2O_2 is reportedly at a intracellular concentration of 70 μM (Meshnick *et al.*, 1977). However H_2O_2 exists transiently in the cell: due to the reactive nature of the molecule it is constantly being synthesised and removed. Assuming that the concentration of H_2O_2 in *Leishmania* is

of H_2O_2 in *Leishmania* is similar, the abundance of thiols means that newly produced molecules will be rapidly reduced and therefore not available to oxidise Sb(III). Moreover enzymes able to mediate the reduction of H_2O_2 have a similar effect: the oxidation of As(III) by H_2O_2 is inhibited when catalase is added to the reaction (Aposhian *et al.*, 2004). Although *Leishmania* lack catalase, they do possess several peroxidases (Vickers *et al.*, 2004; Adak and Datta, 2005) which perform the same function as catalase and are therefore also likely to inhibit Sb(III) oxidation. However, this does not take into account the possibility that Sb(III) could be localised in an area of the parasite that contains higher levels of H_2O_2. Indeed it has recently been shown that when *L. donovani* are treated with Sb(III), intracellular GSH and $T(SH)_2$ are depleted due to efflux of the molecules and inhibition of trypanothione reductase, the enzyme responsible for maintaining the thiols in their reduced state (Wyllie *et al.*, 2004). Similar effects were observed when human macrophages were treated with Sb(III), as were increased level of reactive oxygen species (Wyllie and Fairlamb, 2006), creating conditions favouring the oxidation of Sb(III). The same effects also occurred in *L. donovani* amastigotes when treated with Sb(V). Although these observations certainly do not provide evidence of Sb(III) oxidation in *Leishmania*, it may be the case that conditions can arise within the parasites that enable some oxidation to occur. Therefore, *in vitro* evidence provided in this chapter that under the right conditions Sb(III) can be oxidised is potentially of great importance, although clearly *in vivo* studies are required to ascertain whether it is indeed a factor in antimonial metabolism.

5 Functional study of *L. major* TDR1

5.1 Introduction

In the previous chapter the expression of TDR1 was described, although the analysis of the recombinant protein (rTDR1), which was carried out by Dr. Helen Denton, was not presented. The biochemical characterisation of rTDR1 *in vitro* was an important step towards understanding the functions of TDR1, including whether the protein is capable of reducing pentavalent antimonials, and if, like the proteins it is similar to, it can catalyse thioltransferase reactions and hence may have a role in protection against oxidative stress. The properties of rTDR1 are summarised below, while a full description is provided in Denton *et al.*, 2004.

Recombinant TDR1, shown by gel filtration analysis to be trimeric, exhibited several interesting activities *in vitro*. In contrast to Tc52 which displays no GSH-conjugation activity (Moutiez *et al.*, 1995), but like human oGST1 (Board *et al.*, 2000), rTDR1 exhibited low-rate conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB), with a specific activity of 1.9 nmol/min/mg/protein. However, no reaction could be detected with the other conjugation substrates ethacrynic acid and 1,2-epoxy-3(4-nitrophenoxy)propane. Like glutaredoxin (GRX), dehydroascorbate reductase (DHAR) and thioltransferase activities were evident, with TDR1 capable of using GSH as an electron donor to reduce both dehydroascorbate and the synthetic disulphide 2-hydroxyethyldisulphide. The specific activities for these two substrates – 11.9 μmol/min/mg/protein and 11.2 μmol/min/mg/protein – were similar, and were significantly higher than those reported for the human oGST protein when assayed under similar conditions (Board *et al.*, 2000). Taken together, these results indicate that the enzymatic capabilities of TDR1 are more similar to oGST and GRX than to other GSTs that typically have high GSH-conjugation activity. The result that TDR1 acts more like GRXs, proteins that share predicted active site sequences with TDR1 but are otherwise much smaller and rather dissimilar, than GSTs was also observed to be the case for human omega GST (Board *et al.*, 2000).

In addition to these activities, TDR1 was also found to be capable of reducing sodium stibogluconate and Glucantime, giving specific activities (μmol/min/mg/protein) of 6.3 and 1.4, respectively. Significantly higher specific activities were recorded with the pentavalent arsenicals sodium arsenate and methylarsonate (MMA^V). The BPR assay (Frezard *et al.*, 2001), which detects trivalent antimonial species, was employed to analyse the product(s) formed in the reactions with pentavalent antimonials. In addition, using the BPR assay, it

was shown that a trivalent antimonial compound was produced in the reaction between TDR1 and the pentavalent antimonial drug, sodium stibogluconate. This was instrumental in determining the relevance of the findings reported above, as it is the reduction of sodium stibogluconate to a trivalent form that reportedly mediates toxicity (Shaked-Mishan *et al.*, 2001).

The fact that rTDR1 is capable of reducing pentavalent antimonials to a trivalent form was clearly of great interest. It is generally accepted that pentavalent antimonial drugs are toxic specifically to amastigotes, yet it is not known if this is due to increased parasite susceptibility or stage-specific reduction – and hence activation – of the pentavalent compounds. Controversy also persists over whether activation occurs in the macrophage or the parasite (Ephros *et al.*, 1999, Sereno *et al.*, 1998). It has been reported that only amastigotes and not promastigotes are capable of reducing pentavalent antimonials and that Pentostam-resistant *L. donovani* parasites were deficient in this activity Shaked-Mishan *et al.*, 2001. Although it has been demonstrated that some thiols are capable of directly reducing pentavalent antimonials (Frezard *et al.*, 2001), the rate is very low and accordingly is unlikely to be as physiologically relevant as the faster, enzyme-mediated reaction reported here. As TDR1 is the first *Leishmania* protein reported to be able of reducing antimonials enzymatically, together with its increased abundance in amastigotes demonstrated in the previous chapter, it is tempting to speculate that TDR1 could play a key role in antimonial susceptibility. However, further analysis is required. These studies have not provided any *in vivo* evidence of the antimonial-reducing capability of TDR1, and the affect this has on the parasite. For example, although reduction is thought to mediate toxicity, it may be possible that upon reduction by TDR1 antimonials are more easily cleared by the cell, making them more quickly detoxified and therefore effectively less toxic. In this chapter, investigations on *L. major* lacking or expressing an increased amount of TDR1 are presented, in an attempt to address whether TDR1 has a bearing on antimonial susceptibility *in vivo*.

There are implications of TDR1 potentially behaving as a GRX in the parasite. Glutaredoxins help maintain the redox balance of the cell by reducing protein and GSH disulphides and have a role in stress-response in the cell (Potamitou *et al.*, 2002). This may point towards the natural role of TDR1 in *Leishmania*: after all, the protein is unlikely to have evolved to improve the efficacy of antileishmanial chemotherapy; that aspect of the protein's function is more likely to be an unhappy coincidence for the parasite. In this chapter, the production and analysis of TDR1 knockout and over-expressing *L. major* parasites is described. One of the important purposes of this was to investigate the cellular

role of TDR1, to elucidate what the protein does regardless of its potential as an antimonial reductase.

5.2 Over-expression of TDR1 in *Leishmania*

It was hoped that manipulating the normal expression levels of TDR1 in *L. major* would result in alterations in the parasites that could be detected. This would enable the importance of TDR1 *in vivo* to be assessed and help establish the protein's functions. The first approach was to increase the expression of TDR1, achieved by over-expressing the protein from an episomal vector.

5.2.1 Creation of TDR1 over-expressing *L. major* promastigotes

The sequence encoding TDR1 was amplified from *L. major* genomic DNA, sub-cloned into the pGEM T-easy and then cloned into the pGL102 plasmid. The plasmid with the *TDR1* gene was named pGL102TDR1; maps of this and the empty vector are shown in figure 5.1. The pGL102 plasmid is a *Leishmania* episomal expression vector and contains the neomycin resistance gene as a selectable marker. Uncut pGL102 and pGL102TDR1 were transfected into *L. major* promastigotes which were subsequently incubated in culture with neomycin so that only successful transformed parasites would grow. The transformed parasite lines will be herein referred to by the names of the vectors they were transformed with (pGL102 and pGL102TDR1).

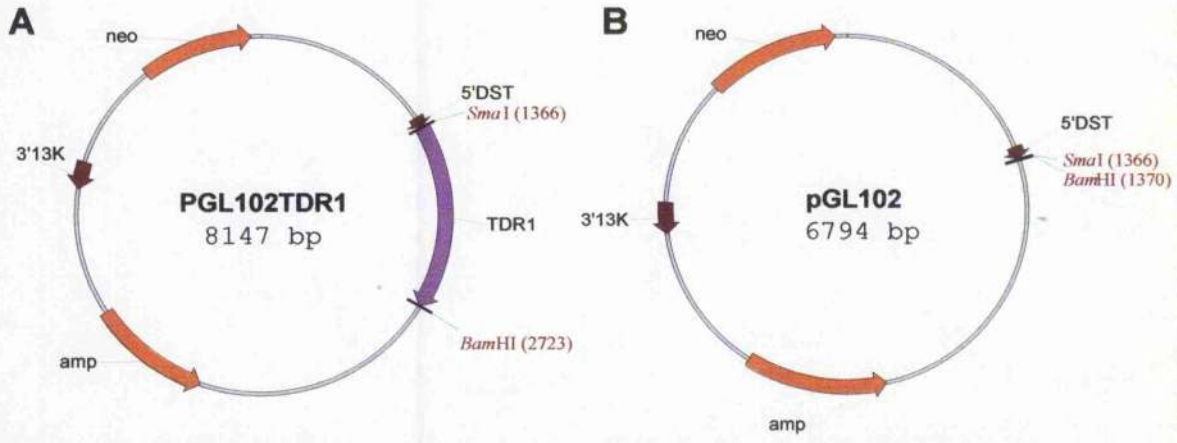

Figure 5.1: Plasmids for the over-expression of TDR1 in *L. major* promastigotes. Schematic representation of the pGL102 plasmid for the over-expression of TDR1 in *Leishmania*. A – pGL102 + TDR1 (pGL102TDR1) for the over-expression of TDR1. amp, ampicillin resistance gene; neo, neomycin resistance gene. The restriction sites used to clone TDR1 into the vector are shown in dark red. B – empty pGL102 vector, for use as a negative control.

5.2.2 Analysis of over-expression

Whether pGL102TDR1 parasites that grew in the presence of the selective drug had been successfully transformed, resulting in increased levels of TDR1, was assessed by western blot analysis. pGL102 and pGL102TDR1 parasites grown with neomycin and in the stationary phase of growth were harvested and lysed and the soluble fractions were retained. The protein concentration of each sample was determined, and equal quantities were separated by SDS-PAGE before western blotting. The sheep ani-TDR1 antibody was used to detect TDR1 expression. To ensure equal loading of the samples, cysteine synthase (CS) expression was examined in parallel with TDR1, using a rabbit anti-CS antibody donated by Dr R. Williams of the University of Glasgow. While the expression of CS remained constant, TDR1 was significantly more highly expressed in pGL102TDR1 than in pGL102 *L. major* promastigotes (figure 5.2); at least a four-fold increase in TDR1 expression was observed.

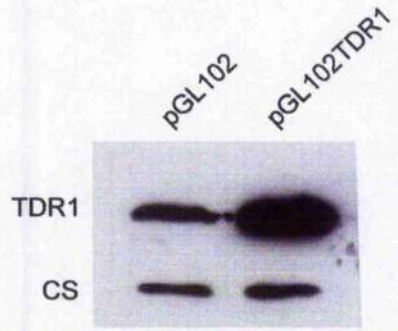

Figure 5.2: Western blot analysis of TDR1 expression in pGL102 and pGL102TDR1 *L. major* promastigotes. 20 µg of soluble parasite lysate was loaded per lane. CS, cysteine synthase protein, used as a control to ensure equal loading. The relative positions of the proteins are indicated to the left of the image.

5.2.3 Phenotypic analysis of TDR1 over-expressing *L. major*

Western blot analysis confirmed that pGL102TDR1 parasites were over-expressing TDR1 as compared to pGL102 parasites, and therefore phenotypic tests could be conducted with confidence that TDR1 was at increased levels. In some experiments the TDR1 over-expressing parasites were compared to WT as well as pGL102 parasites to help corroborate any differences between data sets.

5.2.3.1 Morphology and growth

Parasites over-expressing TDR1 were examined microscopically and no morphological differences could be seen between them and the parasites containing the empty pGL102 vector or WT parasites. After several passages with neomycin present in the culture media, the pGL102 and pGL102TDR1 lines were counted and diluted to the same concentration. Thereafter their growth was monitored by daily counting (figure 5.3). The parasite lines were found to grow at similar rates.

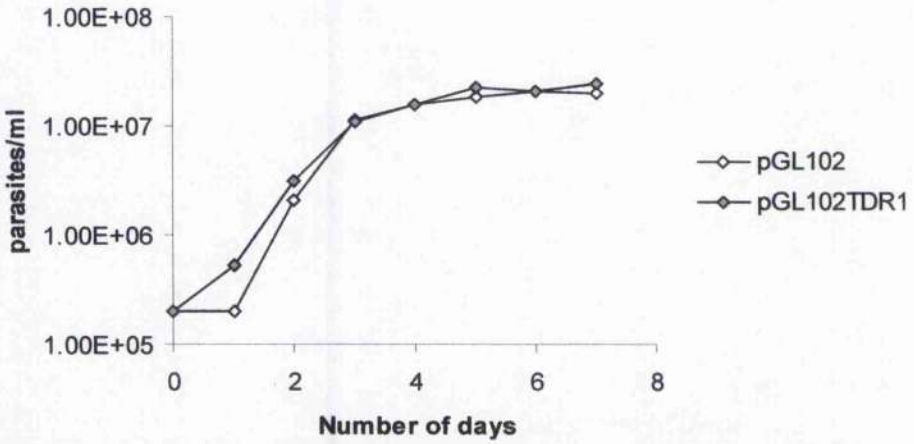

Figure 5.3: Growth curve of pGL102 and pGL102TDR1 *L. major* promastigotes. Cultures were seeded at a concentration of 2 x 10^5 parasites/ml and were counted daily. Both lines were grown with 50 µg/ml neomycin.

5.2.3.2 *In vitro* infectivity to macrophages

The ability of WT, pGL102 and pGL102TDR1 stationary phase promastigote parasite lines to infect peritoneal macrophages was assessed (figure 5.4). pGL102TDR1 infected a higher percentage of macrophages than either the pGL102 or WT lines: approximately 4 times and twice as many, respectively. This suggests that the over-expression of TDR1 in parasites aids infection. However, the differences were not significant which reflects the low number of replicates of this experiment that were performed: further analysis is required to conclude whether over-expression of TDR1 impacts upon the infectivity of the parasites to macrophages. The variation between the infection rates between the WT and pGL102 lines was also of concern as they would be expected to infect at a similar rate. It is possible that the pGL102 line have been adversely affected by the transformation procedure, causing them to be less infective *in vitro*.

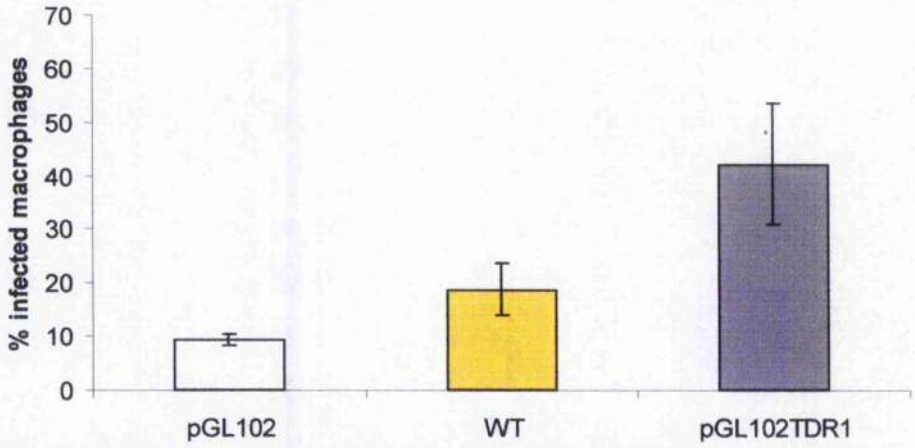

Figure 5.4: Infectivity of WT, pGL102 and pGL102TDR1 *L. major* promastigotes to macrophages. Stationary phase promastigotes were used to infect peritoneal macrophages at a rate of 2:1 and the slides were incubated for 5 days post-infection. Results are the means ± SE from at least two experiments. The infection rates of the different parasite lines were not significantly different ($p > 0.05$)

5.2.3.3 *In vivo* infectivity to mice

To assess the *in vivo* infectivity, metacyclic pGL102 and pGL102TDR1 parasites were purified from stationary phase cultures and inoculated into mouse footpads, the thicknesses of which were then measured over time (figure 5.5). pGL102TDR1 parasites were responsible for significantly more rapid footpad growths than those that were induced by pGL102. However, footpad infectivity experiments described elsewhere in this chapter suggest that the infectivity of pGL102 parasites may be compromised: footpads infected with WT parasites reach an average of 4 mm after 6 weeks (figure 5.16), while here footpads infected with pGL102 parasites are an average of just 3.5 mm after 10 weeks. However, although the experimental conditions are the same between these two experiments, some parameters may be subject to change e.g. the amount of time between metacyclic promastigote purification and inoculation. Therefore the differences observed between the two experiments may not be due to the pGL102 line being defective. Regardless of whether pGL102 parasites are infectious as WT, the footpad infections caused by pGL102TDR1 are particularly virulent as after just five weeks the footpads had reached an average width of 4.9 mm. The difference between the footpad infections caused by the pGL102TDR1 parasites in this experiment and the WT parasites in the experiment described in section 5.3.3.4 and demonstrated in figure 5.16 is significant ($p < 0.02$). As described in the previous section, pGL102TDR1 parasites were also more infective to macrophages than both WT and pGL102 parasites.

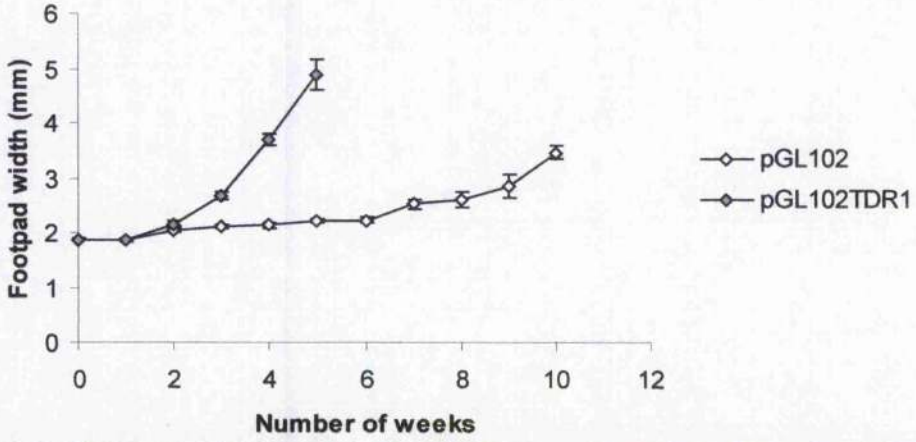

Figure 5.5: Infectivity of pGL102 and pGL102TDR1, *L. major* metacyclic promastigotes to mice. 1 x 10^5 metacyclic promastigotes were resuspended in 20 µl PBS and inoculated into one footpad of each BALB/C mouse. The footpad thicknesses were subsequently measured weekly, and the results are the means ± SD from five mice. The footpad thicknesses of mice infected with pGL102TDR1 parasites were significantly greater than those of mice infected with pGL102 five weeks post-infection ($p < 0.0006$).

5.2.3.4 Effect of Sb(V) on macrophage infections

The effect of sodium stibogluconate (Sb(V)) on macrophages infected with pGL102 and pGL102TDR1 parasites was assessed (figure 5.6). The parasites and macrophages were incubated overnight, the excess parasites were washed off and dilutions of Sb(V) in media, or drug-free media, were applied. The parasites were incubated with the drug for a total of five days, with the drug being refreshed once during that time. At the highest drug concentration (250 μg/ml) the percentage of macrophages infected by the pGL102 and pGL102TDR1 parasites had fallen to around 30% of the percentage of cells infected when no drug was applied. There was no significant difference in the effect of Sb(V) between the different parasite lines.

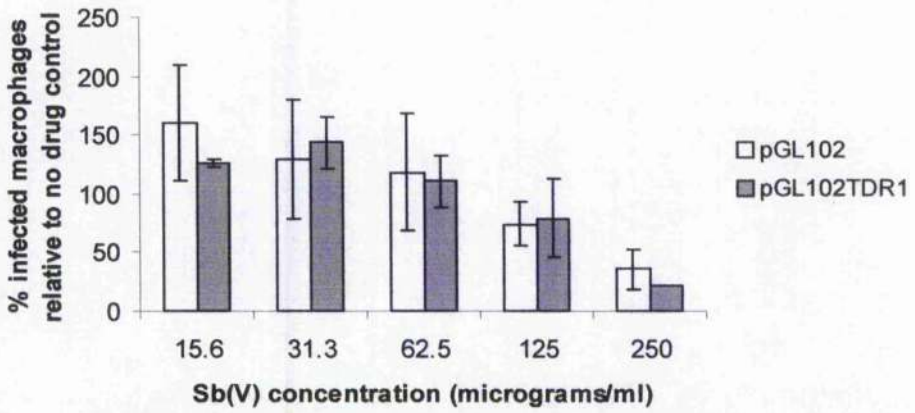

Figure 5.6: Effect of sodium stibogluconate on the infectivity of pGL102 and pGL102TDR1 *L. major* promastigotes to macrophages. Stationary phase promastigotes were used to infect peritoneal macrophages at a rate of 3:1 at 37 °C. After the parasites and macrophages had been incubated together overnight, the parasites were washed off and doubling dilutions of sodium stibogluconate (Sb(V)) were applied. Results are the means ± SE from at least two experiments. There is no significant difference between the effect of Sb(V) on the infectivity of pGL102 and pGL102TDR1 promastigotes to macrophages ($p > 0.5$). The results are expressed as the percentage of cells infected when no drug was present in order to normalize varying levels of infection.

5.2.3.5 IC_{50} values when incubated with agents that induce oxidative stress

IC_{50} values – the concentration of a substance that causes half of the maximum inhibitory effect it exerts to occur – were obtained for H_2O_2 and paraquat, against WT and pGL102TDR1 parasites, and are presented in table 5.1. These were obtained by incubating parasites with doubling dilutions of the compounds in 96-well plates, and adding Alamar Blue to indicate living parasites. H_2O_2 itself is a reactive oxygen species (ROS) while

paraquat can diffuse across cell membranes and react with dioxygen, resulting in the intracellular production of superoxide (O_2^-) (Hassan and Fridovich, 1978). As described in the introduction to this chapter, TDR1 displays glutaredoxin-like activities *in vitro*, functioning as a thioltransferase and dehydroascorbate reductase. Therefore increasing the abundance of TDR1 *in vivo* may be expected to protect the parasites against oxidative stress, and increase their tolerance to agents that induce it. However, the IC50 values obtained for H_2O_2 and paraquat were similar for both WT and pGL102TDR1 parasites. The pGL102TDR1 parasites are slightly more tolerant to H_2O_2 than WT, although paradoxically they are slightly more sensitive to paraquat.

	WT	pGL102TDR1
H_2O_2	253.3 ±2.9	301.7 ±7.6
paraquat	5500 ±1004	2200 ±130

Table 5.1: IC_{50} values of paraquat and H_2O_2 against WT and pGL102TDR1 *L. major* promastigotes. All values were obtained using the Alamar Blue test; parasites were incubated with doubling dilutions of the compounds for a total of four days. The values and standard deviations were calculated using the Grafit software IC_{50} programme. All values are in μM.

5.2.3.6 Growth of promastigotes with paraquat

To further assess the effect of paraquat on pGL102TDR1 parasites compared to *L. major* promastigotes with normal TDR1 levels, parasite cultures were grown with the compound (figure 5.7). WT, pGL102 and pGL102TDR1 promastigotes were seeded at 1 x 10^6 and in standard media containing 5 mM paraquat. Over the course of seven days, the parasite lines grew at similar rates, although between day 3 and day 6 the pGL102TDR1 were at a slightly lower concentration than the WT and pGL102 parasites.

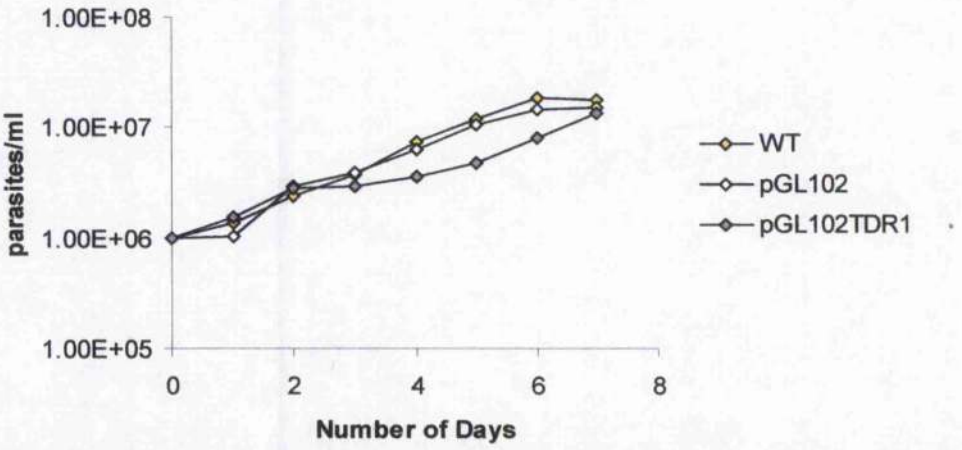

Figure 5.7: Effect of paraquat on the growth of WT, pGL102 and pGL102TDR1 *L. major* promastigotes. The parasites were seeded at 1 x 10^6/ml with 50 μg/ml neomycin and 5 mM paraquat and were counted daily.

5.3 Knocking-out of TDR1 in *L. major*

It was anticipated that removing the *TDR1* gene from *L. major* by two rounds of homologous recombination would affect the parasites and result in a phenotype that would clarify the role of TDR1 *in vivo*. Analysis of the knockout parasites' susceptibility to Sb(V) would also elucidate the role, if any, of TDR1 in pentavalent antimonial activation.

5.3.1 Creation of *L. major TDR1* knock-out parasite lines

The 3' and 5' flanking regions of TDR1 were amplified from *L. major* genomic DNA, sub-cloned into pET28a(+) and cloned into vectors pGL345, pGL1033 and pGL842 which contain hygromycin, bleomycin and blasticidin resistance genes, respectively (figure 5.8). The constructs containing the flanking regions and drug resistance markers were cut out and purified and the linear DNA was used to transform *L. major* promastigotes. Following the two independent second-round transfections and overnight recovery period, the appropriate selective drugs were added and the cultures were divided: 1 ml of each was used to set up serial dilutions in an attempt to derive clonal lines. Two independent lines, KOTDR11 and KOTDR18, were generated, both having undergone transformation with two constructs sequentially and grown up in the presence of the selection drugs. KOTDR11 was transformed with DNA obtained from the pGL345 (hygromycin) and pGL1033 (bleomycin) plasmids, while KOTDR18 was generated with DNA from the pGL345 and pGL842 (blasticidin) plasmids. In addition, a clonal line was derived from the initial serial dilutions of the KOTDR1 population; it was named KOTDR11A. After several passages in the selective drugs, three further clonal lines were derived by setting up KOTDR11 and KOTDR18 in 96-well plates at a concentration of approximately 0.1 parasites per well. One clone, KOTDR11B, was isolated from KOTDR11 and two clones were isolated from KOTDR18: KOTDR18A and KOTDR18B. The clones were derived in the presence of selective drugs, but these were omitted from the media following the isolation of the clones.

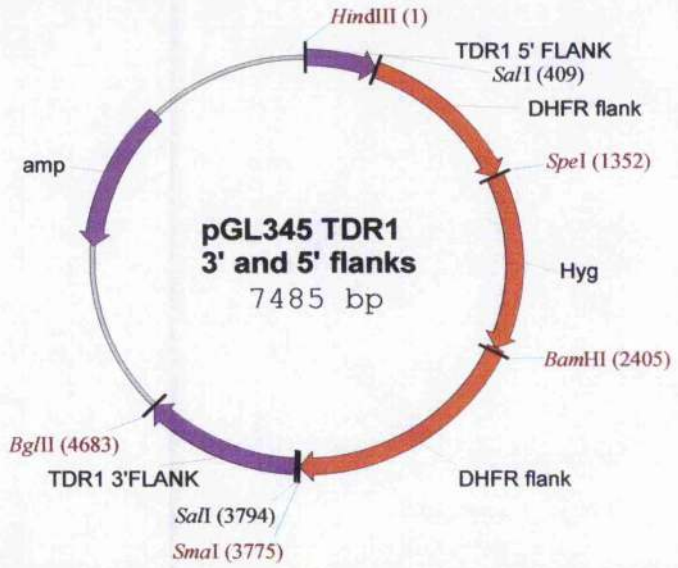

Figure 5.8: Construct for the gene knock-out of TDR1 in *L. major* promastigotes. Schematic representation of the pGL345 plasmid containing the TDR1 3' and 5' flanking regions. The second plasmids used for the construction of the TDR1 knock-out lines were pGL842 and pGL1033, which are exactly the same as pGL345 except that they contain blasticidin and bleomycin resistance genes respectively, rather than hygromycin resistance gene. Hyg, hygromycin resistance gene; amp, ampicillin resistance gene; DHFR flanks, flanking regions of the dihydrofolate reductase gene. The restriction sites used to clone the TDR1 flanks and hygromycin resistance gene into the vector are shown in dark red. *HindIII* and *BglII* were used to cut out the flank-containing linear construct for transfection into *L. major*.

5.3.2 Analysis of knock-out lines

Whether the *TDR1* gene had successfully been knocked out of KOTDR11A, KOTDR11B, KOTDR18A and KOTDR18B, resulting in the absence of TDR1, was assessed by western blot analysis. WT and TDR1 knockout parasites in the stationary phase of growth were harvested and lysed and the soluble fractions were retained. The protein concentration of each sample was determined, and equal quantities were separated by SDS-PAGE before western blotting. The sheep ani-TDR1 antibody was used to detect TDR1 expression. To ensure equal loading of the samples and as a positive control, CS expression was examined in parallel with TDR1, using the rabbit anti-CS antibody as before. While CS expression was apparent in all lines tested, TDR1 was solely expressed in WT parasites (figure 5.9). Therefore KOTDR11A, KOTDR11B, KOTDR18A and KOTDR18B were judged as being successful TDR1 knockouts.

Figure 5.9: Western blot analysis of TDR1 expression in WT, KOTDR18A, KOTDR18B, KOTDR11A and KOTDR11B *L. major* promastigotes. 15 µg of soluble parasite lysate was loaded per lane. CS, cysteine synthase protein, was used as a control to ensure equal loading. The relative positions of the proteins are indicated to the left of the image.

5.3.3 Phenotypic analysis of TDR1 knock-out lines

5.3.3.1 Morphology and growth

TDR1 knockout parasites were examined microscopically and no morphological differences could be seen between them and WT. The KOTDR11A, KOTDR11B, KOTDR18A and KOTDR18B clonal lines were counted and diluted to the same concentration. Thereafter their growth was monitored by counting at regular intervals (figure 5.10). The KOTDR18A and KOTDR11B lines took slightly longer to reach stationary phase than the other lines. KOTDR18A did not reach a density greater than 1.2 x 10^7 parasites/ml, while all other lines reached 2 x 10^7 parasites/ml. Moreover, after 30 days in culture, the KOTDR18A parasites began to die while the other lines did not. After the KOTDR11A, KOTDR11B and KOTDR18B lines had been passed through mice, the growth curves were repeated and all three grew at similar rates to, and reached similar densities in the stationary growth phase as WT parasites.

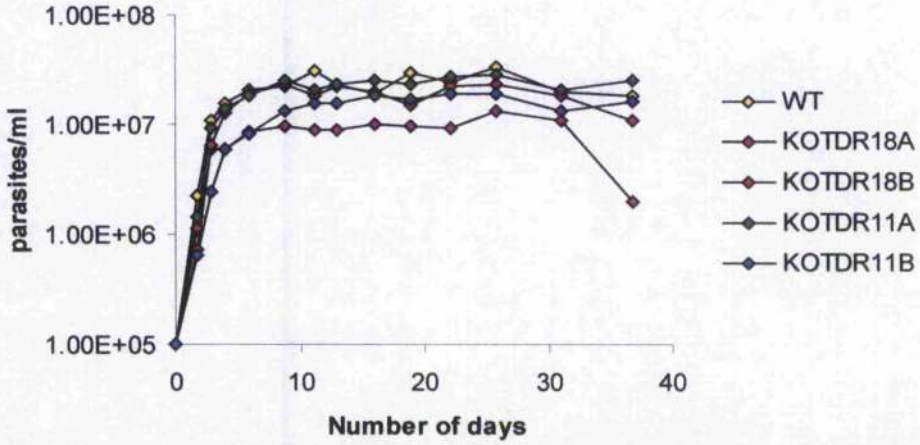

Figure 5.10: Growth curve of WT, KOTDR11A, KOTDR11B, KOTDR18A and KOTDR18B *L. major* promastigotes. Cultures were seeded at a concentration of 1 x 10^5 parasites/ml and were counted at regular intervals. All parasite lines were grown without any selective drugs added.

5.3.3.2 Differentiation to metacyclic promastigotes

The percentage of WT, KOTDR11A, KOTDR11B, KOTDR18A and KOTDR18B promastigotes that differentiated to metacyclic forms was monitored over time (figure 5.11). Parasite cultures were seeded at equal concentrations and, after they had reached the stationary phase of growth, aliquots were removed and the percentage of metacyclic forms in each sample was assessed. The percentage of metacyclic forms in each of the parasite lines tested increased over time. The percentage of metacyclic forms in each culture was similar at each time interval, although the highest percentage was consistently in the KOTDR11A line. At 16 days after the cultures were initiated, a lower percentage of metacyclogenesis was observed in the KOTDR18A sample, as compared to the other lines.

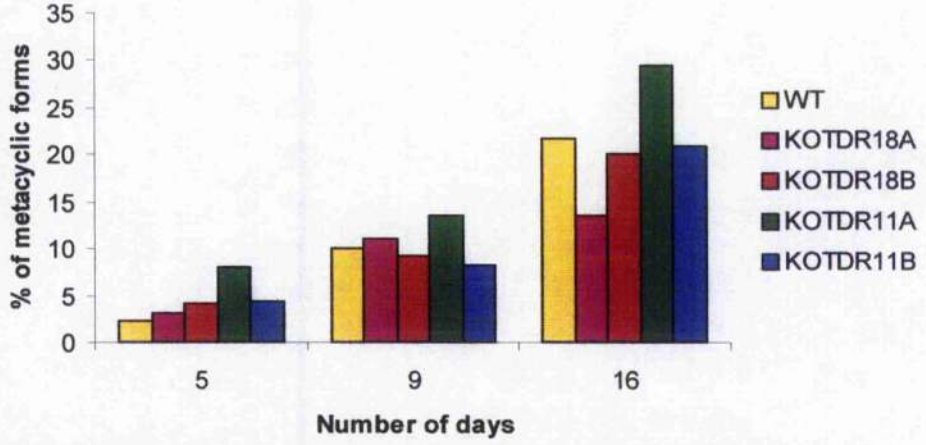

Figure 5.11: Metacyclic formation in WT, KOTDR11A, KOTDR11B, KOTDR18A and KOTDR18B *L. major* promastigotes over time. Parasite cultures were seeded at 1 x 10^5/ml and metacyclogenesis was assessed after they had reached the stationary phase of growth. The parasites in each culture were counted, incubated with peanut agglutinin and re-counted. Metacyclogenesis was also assessed by analysis of parasite morphology. Number of days refers to the time since the cultures were initiated.

5.3.3.3 *In vitro* infectivity to macrophages

The ability of WT, KOTDR18B, KOTDR11A and KOTDR11B stationary phase promastigote parasite lines to infect peritoneal macrophages was assessed (figure 5.12). The KOTDR18A line was not used here, or in any other macrophage infectivity experiments because, as discussed in section 5.3.3.4, this line was not infective to mice. While the KOTDR18B, KOTDR11A and KOTDR11B parasite infected macrophages at similar levels, the WT parasites infected a significantly lower number of cells. This suggests that the loss of TDR1 enhances the macrophage infectivity of *L. major*. This was

an unexpected result, as the over-expression of TDR1 in *L. major* promastigotes had a similar effect (section 5.2.3.2).

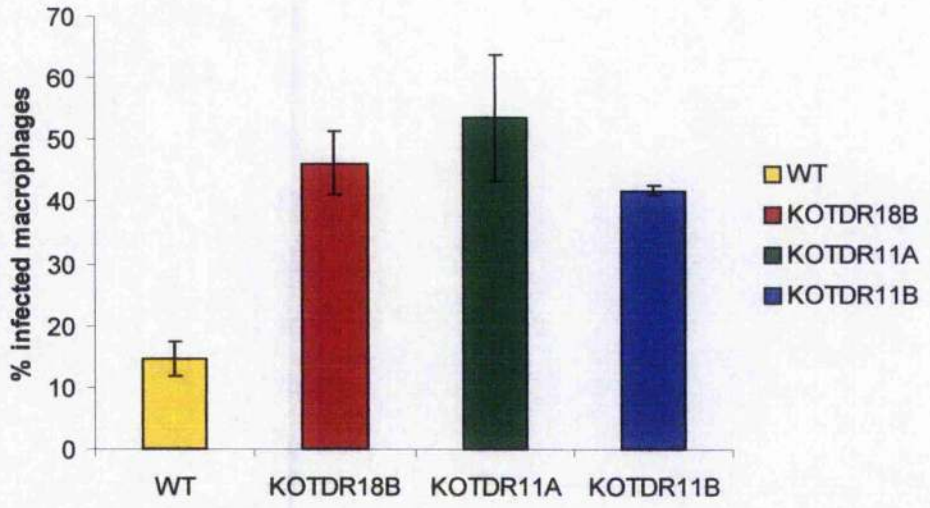

Figure 5.12: Infectivity of WT, KOTDR11A, KOTDR11B, KOTDR18A and KOTDR18B *L. major* promastigotes to macrophages. Stationary phase promastigotes were used to infect peritoneal macrophages at a rate of 2:1 and the slides were incubated for 6 days at 37 °C post-infection. Results are the means ± SE from at least two experiments. All three TDR1 knockout lines are significantly more infectious to macrophages than WT parasites ($p < 0.05$).

In the previous experiment the macrophages were incubated at 37 °C. Whether the incubation temperature affected the infectivity of WT, KOTDR18B, KOTDR11A and KOTDR11B promastigotes was assessed (figure 5.13A). At both 35 °C and 35 °C, the WT parasites infected a lower percentage of macrophages than the TDR1 knockout lines. However, this effect was much more pronounced at 37 °C: while the percentage of infected macrophages for the three TDR1 knockout lines remained constant between the two temperatures, the WT parasites infected less than half the percentage of macrophages at 37 °C than they did at 35 °C. The effect of the different temperatures on the average number of parasites in each infected cell was also determined (figure 5.13B). When incubated at 35 °C the average number of parasites in each infected cell varied between the KOTDR18B, KOTDR11A and KOTDR11B lines (approximately 11, 14 and 17, respectively). However these were all higher than the average of 8 parasites per cell observed in macrophages infected with WT parasites. At 37 °C there was a decrease in the average number of parasites per cell in all the lines tested. At the higher temperature the average number of parasites was similar in cells infected by the three TDR1 knockout lines, and once again the number of WT parasites per cell was considerably less.

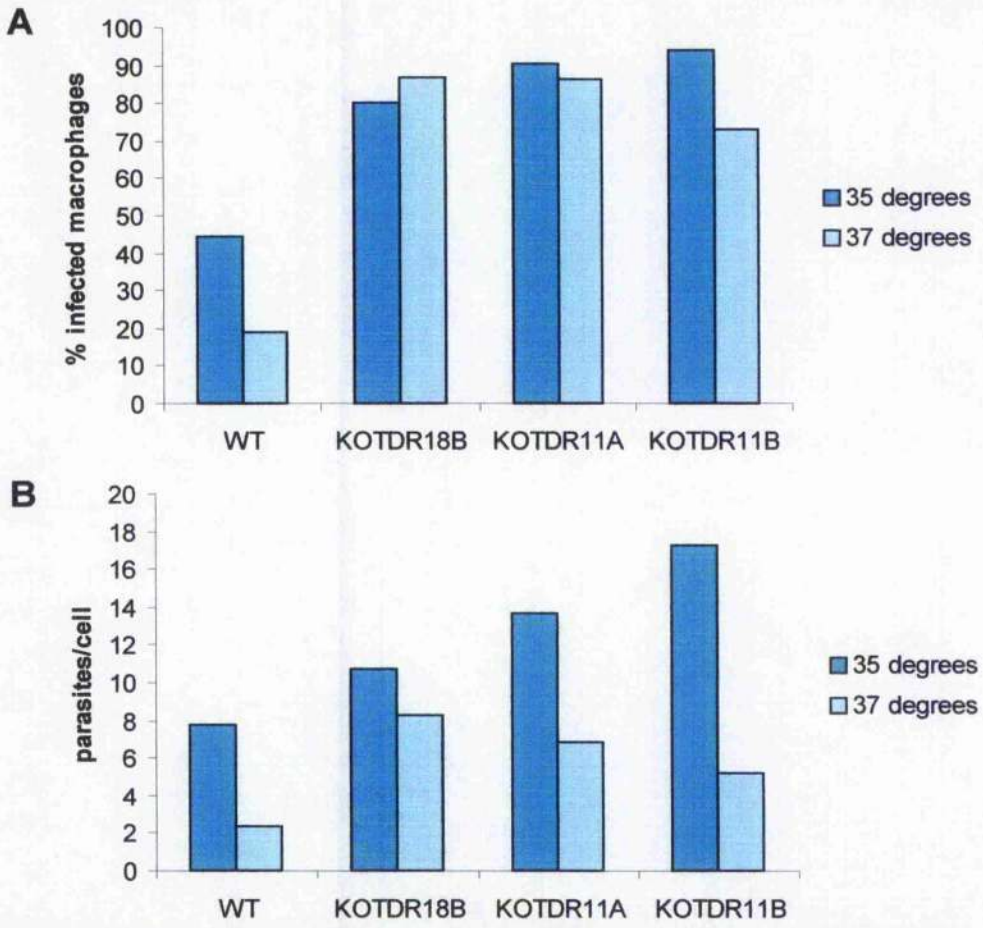

Figure 5.13: Infectivity of WT, KOTDR11A, KOTDR11B, KOTDR18A and KOTDR18B *L. major* promastigotes to macrophages and the number of parasites per infected cell, when incubated at different temperatures. Stationary phase promastigotes were used to infect peritoneal macrophages at a rate of 3:1 and the slides were incubated for 6 days post-infection. A – the percentage of macrophages infected after 6 days. B – the average number of parasites present in each in infected cell. The number of parasites present in at least 20 infected cells was counted, and the average number calculated.

A second experiment was conducted to analyse the affect of temperature, but also the infection ratio of parasites to cells, on the infectivity of WT, KOTDR11A, KOTDR11B, KOTDR18A and KOTDR18B to macrophages (figure 5.14). A second *L. major* WT line with a higher passage number was also used, to corroborate the observation in the previous experiments that the WT parasites were less infective to macrophages than the TDR1 knockout lines. Parasites with a higher passage number were used as these had not been so recently harvested from animals and had had longer to adjust to growing *in vitro*. The percentage of infected macrophages by the WT lines were highly similar when infection ratios of 2:1 and 3:1 parasites to macrophages were used, and at both 35 °C and 37 °C. Both WT lines infected a lower percentage of macrophages than the three TDR1 knockout lines, which infected similar levels of cells. In all the parasite lines used in these experiments and at both temperatures, a significantly higher percentage of macrophages

were infected when the infection ratio of parasites to cells was 3:1 as opposed to 2:1. Once again, the percentage of macrophages infected by WT parasites was significantly lower at 37 °C than 35 °C; this effect was apparent with both WT lines used, and was regardless of the infection ratio used. The percentages of macrophages infected with the three TDR1 knockout lines at each of the infection ratios used were not significantly different between the two temperatures.

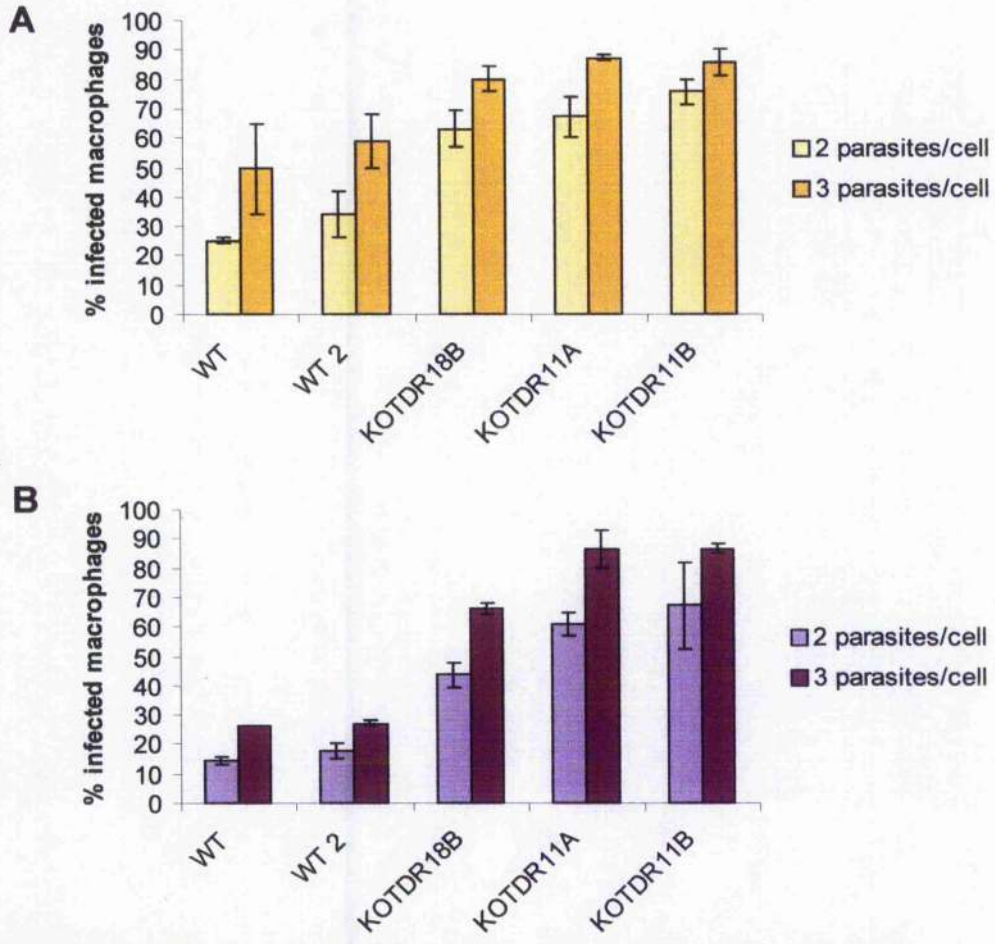

Figure 5.14: Infectivity of two independent WT lines and KOTDR11A, KOTDR11B, KOTDR18A and KOTDR18B *L. major* promastigotes to macrophages using different infection ratios and when incubated at different temperatures. Stationary phase promastigotes were used to infect peritoneal macrophages at a rate of 2:1 or 3:1 as indicated, and the slides were incubated for 6 days post-infection. A – incubated at 35 °C. B – incubated at 37 °C. Results are the means ± SE from at least two experiments. For both WT and TDR1 knockout parasites and at both temperatures, the percentage of cells infected when an infection ratio of 3:1 was used was significantly higher than when an infection ratio of 2:1 was used ($p < 0.05$). For each infection ratio the percentage of macrophages infected with WT parasites when the cells were incubated at 35 °C was significantly higher than when incubated at 37 °C ($p < 0.05$). This was not the case for the TDR1 knockout parasites ($p > 0.1$).

Finally, the effect of time on macrophage infections by both WT lines and the three TDR1 knockout lines was examined. The parasites were removed after being incubated with the macrophages overnight, and the cells were incubated for a further one, three or five days before the percentage of infected cells was assessed (figure 5.15). After one day, all the parasite lines resulted in a similar percentage of infected macrophages. However, by the fifth day both the WT lines infected significantly less cells than the three TDR1 knockout lines, which again infected very similar levels of macrophages.

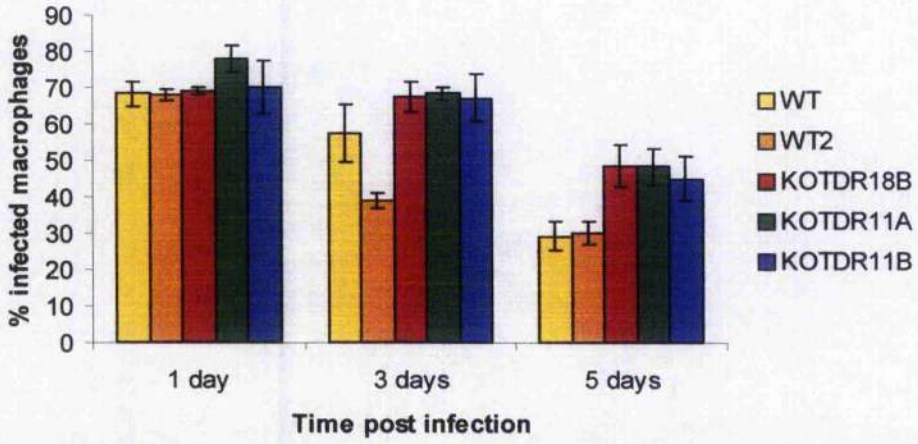

Figure 5.15: Infectivity of two independent WT lines and KOTDR11A, KOTDR11B, KOTDR18A and KOTDR18B *L. major* promastigotes to macrophages over time. Stationary phase promastigotes were used to infect peritoneal macrophages at a rate or 3:1 and the slides were incubated for 1, 3 or 5 days post-infection as indicated. Results are the means ± SD from at least three experiments. After five days the percentage of macrophages infected by the WT parasites was significantly less than the percentage infected by the TDR1 knockout lines ($p < 0.05$).

Taken together, these experiments show that all three TDR1 knockout lines consistently infect a significantly higher percentage of macrophages than WT parasites do, and also produce infections with a higher number of parasites per infected cell than WT. The WT cell lines infected a lower percentage of cells when incubated at 37 °C as compared to 35 °C; this effect was not apparent in infections by the TDR1 knockout lines. A similar phenomenon was observed when the macrophages were incubated for varying amounts of time post-infection: while all lines produced similar levels of infected macrophages after one day, after five days the percentage of macrophages infected with the WT parasites was significantly less than the percentage infected with the TDR1 knockout lines. The three TDR1 knockout lines behaved similarly, causing comparable levels of cells to be infected under all the conditions tested here. The two WT lines used also produced similar results.

For all the lines tested and at both 35 °C and 37 °C, a 3:1 infection ratio resulted in a higher percentage of infected macrophages that when a 2:1 infection ratio was used.

5.3.3.4 *In vivo* infectivity to mice

To assess the *in vivo* infectivity of the TDR1 knockout parasites, metacyclic promastigotes were purified from stationary phase cultures and inoculated into mouse footpads, the thicknesses of which were then measured over time. Initially only the KOTDR11A and KOTDR18A lines were used, together with WT parasites (figure 5.16). Both the WT and KOTDR11A parasites infected the mice footpads, with the KOTDR11A parasites causing slightly more rapid footpad growths to occur than the WT parasites. However, the KOTDR18A line was found to be completely non-infective to the mice; the footpad widths did not increase in any of the mice. The same result was obtained in a second experiment using this line. This was an unexpected result given the infectivity of the KOTDR11A line. However, previous experiments described in section 5.3.3.1 show that KOTDR18A promastigotes did not grow to as high densities as WT and the other TDR1 knockout lines, and expired before them. Metacyclogenesis was also reduced in the KOTDR18A line, albeit only at the latest time point (section 5.3.3.2). Therefore the KOTDR18A line was judged to have been damaged in some way – possibly during the transformation procedure – and was therefore not used for subsequent phenotypic analysis.

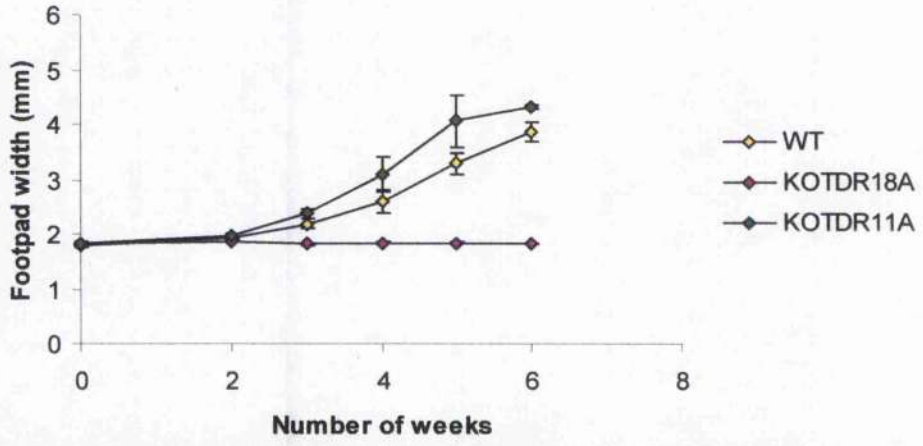

Figure 5.16: Infectivity of WT, KOTDR18A and KOTDR11A *L. major* metacyclic promastigotes to mice. 1 x 10^5 metacyclic promastigotes were resuspended in 20 µl PBS and inoculated into one footpad of each BALB/C mouse. The footpad thicknesses were subsequently measured weekly, and the results are the means ± SD from three mice. The footpad thicknesses of mice infected with KOTDR11A parasites were significantly greater than those of mice infected with WT parasites five weeks post-infection ($p < 0.025$).

The infectivity of WT parasites and the remaining TDR1 knockout lines KOTDR11A, KOTDR11B and KOTDR18B to mice footpads was investigated (figure 5.17). All the lines infected the mice, although the three TDR1 knockout lines caused significantly more rapid footpad width increases to occur than the WT parasites did. This reflects the *in vitro* data, with the TDR1 knockout lines being consistently being more infective to macrophages than the WT parasites. Following the experiment, parasites were recovered from the footpads and cultured as normal for use in further investigations.

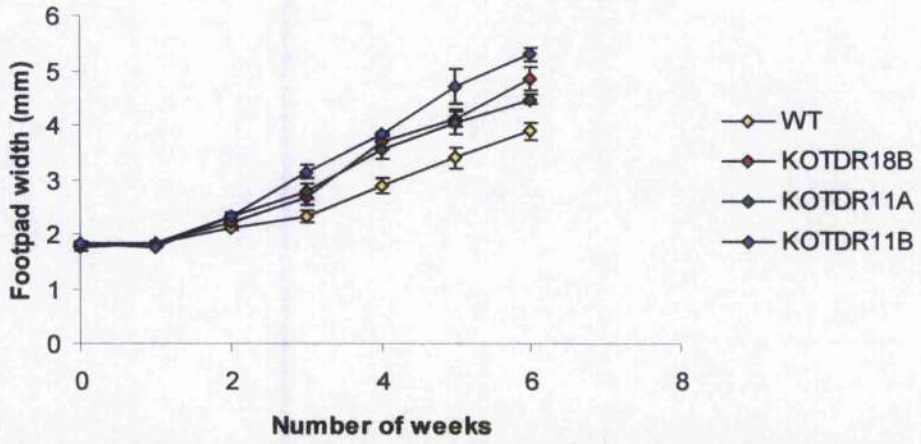

Figure 5.17: Infectivity of WT, KOTDR18B, KOTDR11A and KOTDR11B *L. major* metacyclic promastigotes to mice. 1 x 10^5 metacyclic promastigotes were resuspended in 20 µl PBS and inoculated into one footpad of each BALB/C mouse. The footpad thicknesses were subsequently measured weekly, and the results are the means ± SD from five mice. The footpad thicknesses of mice infected with all three TDR1 knockout lines were significantly greater than those of mice infected with WT parasites five weeks post-infection ($p < 0.05$).

5.3.3.5 Effect of Sb(V) on infectivity to macrophages

The effect of Sb(V) on macrophages infected with WT, KOTDR11A, KOTDR11B and KOTDR18B parasites was assessed, as before. Initially only the WT and KOTDR11A lines were used (figure 5.18). The results are presented as both the percentage of infected macrophages at the varying drug concentrations, and as of the percentage of the level of infection achieved when no drug was applied; this is to normalise the data for the anomalies in the infection levels between the lines. At the second-highest drug concentration (250 µg/ml) the percentage of macrophages infected with WT parasites had fallen to around 50% of the percentage of cells infected when no drug was applied, while the infection level of macrophages infected with the KOTDR11A line remained unchanged. This was the only drug concentration at which a significant difference was observed and suggests that macrophage infections of KOTDR11A may be less susceptible

to Sb(V) than WT infections. This is in accordance with the hypothesis that TDR1 is able to reduce pentavalent antimonials to a more harmful trivalent form *in vivo*, effectively mediating toxicity of the drug. However, the large standard errors obtained from the data, caused by inconsistency between duplicate experiments, meant that further analysis was required.

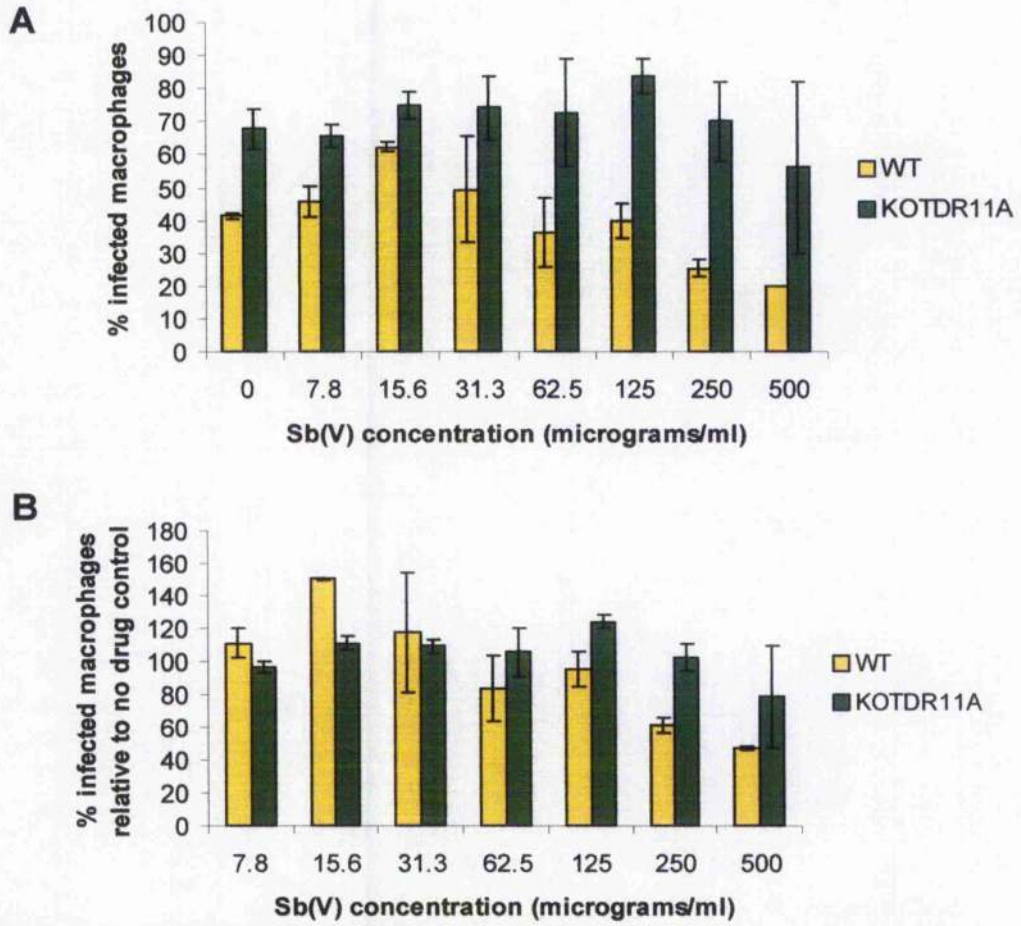

Figure 5.18: Effect of sodium stibogluconate on the infectivity of WT and KOTDR11A *L. major* promastigotes to macrophages. Stationary phase promastigotes were used to infect peritoneal macrophages at a ratio of 3:1. Doubling dilutions of sodium stibogluconate (Sb(V)) were applied as before and cells were incubated for 5 days with the drug. A - % infected macrophages. B – results expressed as the percentage of cells infected when no drug was present in order to normalize varying levels of infection. Results are the means ± SE from two experiments. When the drug concentration was 250 µg/ml, the percentage of macrophages infected with WT parasites relative to the no drug control was significantly less than the percentage infected with KOTDR11A parasites ($p < 0.04$). At all other drug concentrations there was no significant difference ($p > 0.05$).

In a second experiment the effect of Sb(V) on macrophages infected with WT, KOTDR11A, KOTDR11B and KOTDR18 was investigated (figure 5.19). Again, the raw data together with the normalised results are presented. In this experiment, the percentage of macrophages infected with WT parasites was very low: only around 15% of cells were infected when no drug was present. Meanwhile the infection levels achieved with the TDR1 knockout lines was consistently around 50%. At the highest drug concentration (1 mg/ml) the percentage of macrophages infected with WT and KOTDR18B parasites had fallen to around 50% and 55%, respectively, of the percentage of cells infected when no drug was applied, while the level of macrophages infected with KOTDR11A and KOTDR11B were approximately 80%. However, there was no consistent significant differences between the effect of Sb(V) on WT infections compared to TDR1 knockout infections. The low infection rate observed for the WT line when no drug was present is problematic when analysing the data: the effect of Sb(V) on WT infection levels may be far more dramatic if the initial infection rates were higher.

The effect of the incubation temperature on macrophages infected with WT, KOTDR11A, KOTDR11B and KOTDR18 parasites, and treated with Sb(V) was assessed. Both the percentage of infected macrophages (figure 5.20) and the average number of parasites per cell (figure 5.21) were analysed. Only the normalised data is presented here (the percentage of the infection levels and parasites/cell observed with no drug present); the results correlate with the data presented in figure 5.13 as they are part of the same experiment. Only two concentrations of Sb(V) were used in these experiments: 0.5 mg/ml and 2.5 mg/ml and at the latter Sb(V) may also be slightly toxic to the macrophages. This high concentration was used to assess whether improved toxicity to *L. major* could be achieved. With all the parasite lines tested, Sb(V) was much more effective at reducing the percentage of infected macrophages at 37 °C than at 35 °C. However, the effect of the drug on the number of parasites per cell did not differ between the two temperatures (although, as described in section 5.3.3.3, the average number of parasites per cell is less at 37 °C than at 35 °C for all the lines tested). No macrophages infected with WT parasites could be detected when the cells had been incubated at 37 °C, although cells were infected with all three TDR1 knockout lines at this temperature. With the exception of this observation, there were no noticeable differences in the effect of Sb(V) on the infections between the cell lines; this applies to both the percentage of infected macrophages and to the average number of parasites per cell. As before, much higher levels of infection were achieved with the TDR1 knockout lines than the WT parasites and this may have affected this inference.

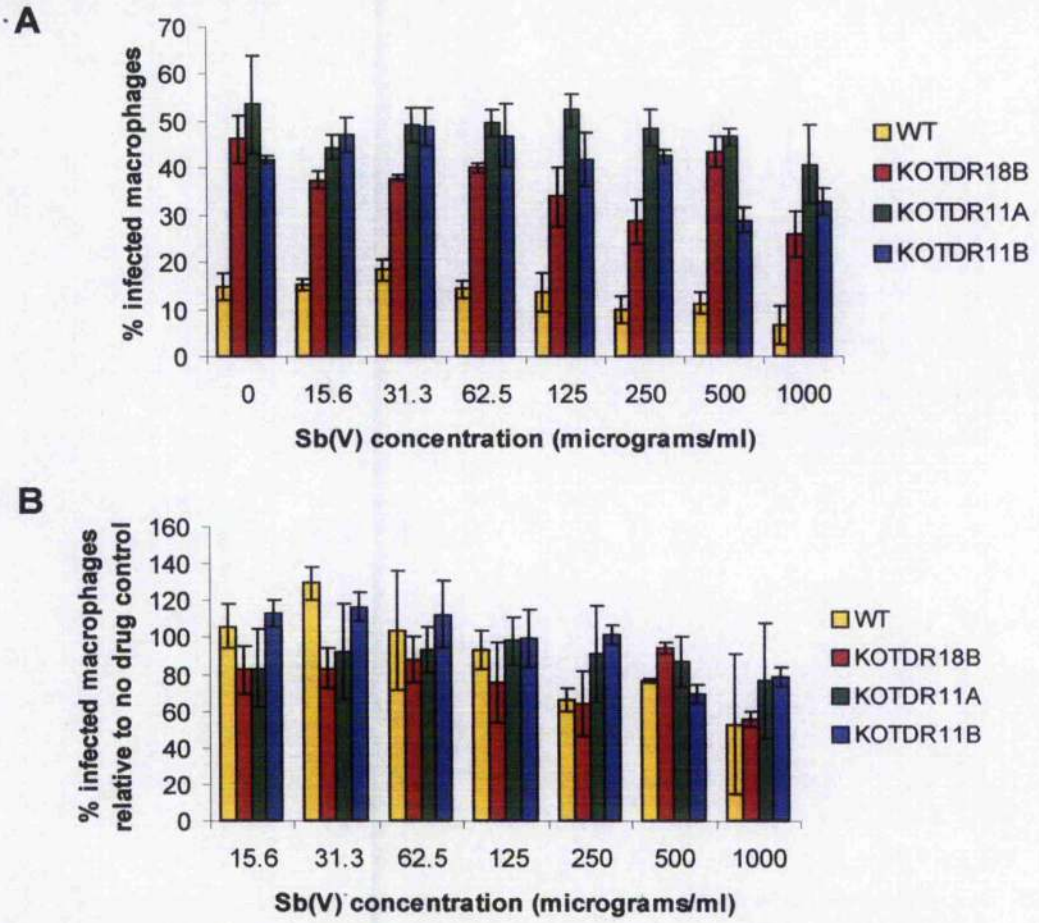

Figure 5.19: Effect of sodium stibogluconate on the infectivity of WT, KOTDR18B, KOTDR11A and KOTDR11B *L. major* promastigotes to macrophages. Stationary phase promastigotes were used to infect peritoneal macrophages at a rate of 2:1. Doubling dilutions of sodium stibogluconate (Sb(V)) were applied as before and cells were incubated for 7 days at 37 °C with the drug. Results are the means ± SE from two experiments. A - % infected macrophages. B - results expressed as the percentage of cells infected when no drug was present in order to normalize varying levels of infection. At all drug concentrations used, there were no significant differences in the effect of Sb(V) on the percentage of macrophages infected with the WT and TDR1 knockout lines relative to the no drug control ($p > 0.05$).

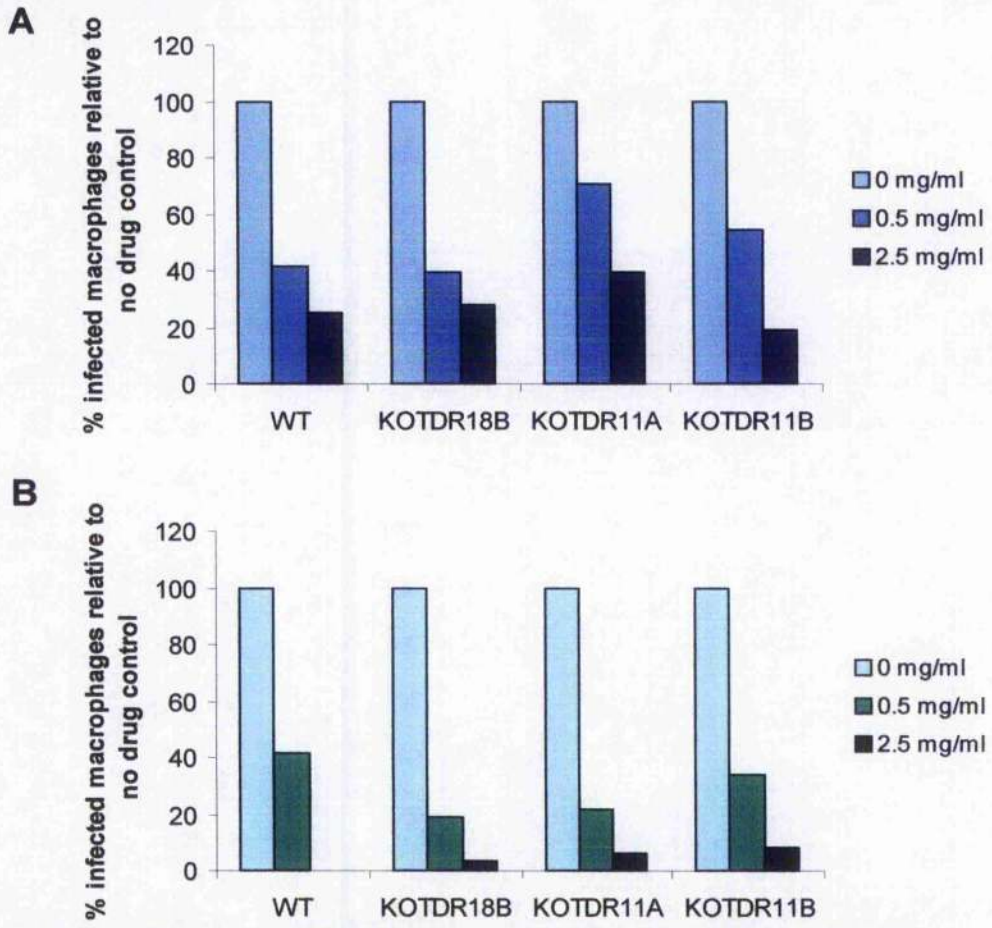

Figure 5.20: Effect of sodium stibogluconate on the infectivity of WT, KOTDR18B, KOTDR11A and KOTDR11B *L. major* promastigotes to macrophages at varying temperatures. Stationary phase promastigotes were used to infect peritoneal macrophages at a rate of 3:1. Sodium stibogluconate (Sb(V)) at one of two concentrations, or no drug, was applied and cells were incubated for 5 days. The results are expressed as the percentage of cells infected when no drug was present in order to normalize varying levels of infection. A – incubated at 35 °C. B – incubated at 37 °C.

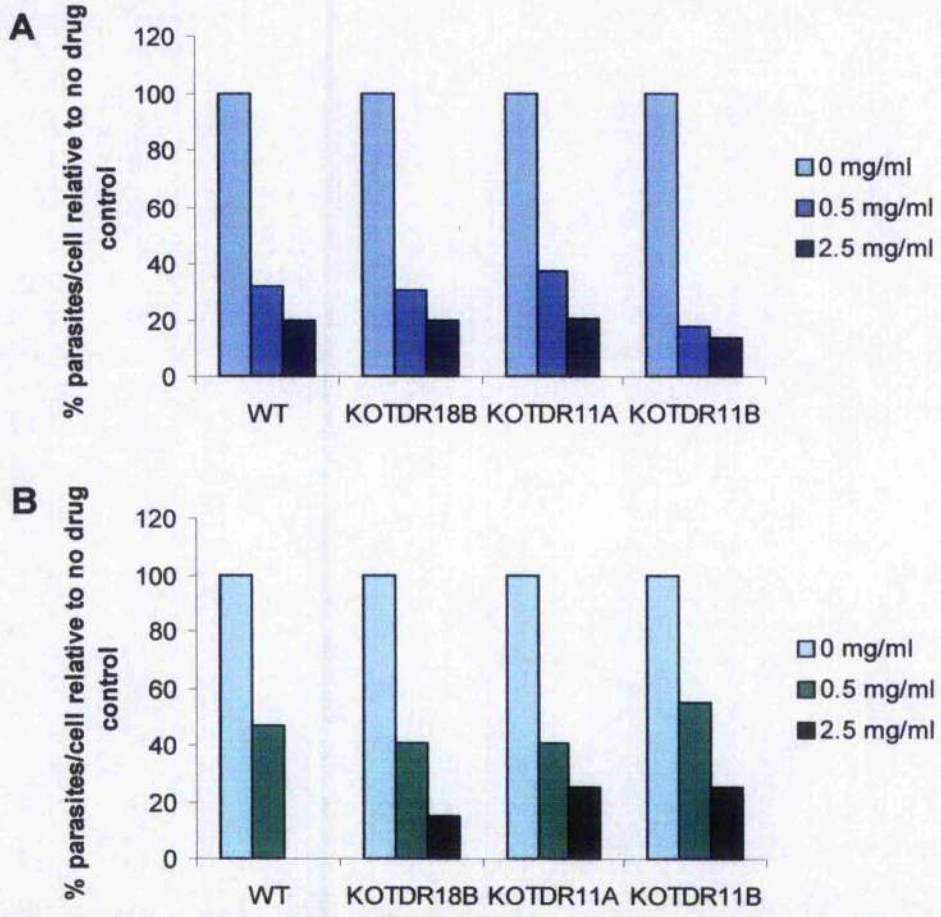

Figure 5.21: Effect of sodium stibogluconate on the average number of parasites per infected macrophage of infections with WT, KOTDR18B, KOTDR11A and KOTDR11B *L. major* promastigotes at varying temperatures. Stationary phase promastigotes were used to infect peritoneal macrophages at a rate of 3:1. Sodium stibogluconate (Sb(V)) at one of two concentrations, or no drug was applied and cells were incubated for 5 days. The number of parasites present in at least 20 infected cells was counted, and the average number calculated. The results are expressed as the percentage parasites per cell that were present when no drug was applied, in order to normalize the data. A – incubated at 35 °C. B – incubated at 37 °C.

5.3.3.6 IC_{50} values when incubated with toxic substances

Using both the acid phosphatase and Alamar Blue assays, IC_{50} values were obtained for various substances against WT and TDR1 knockout parasites. Three different hydroperoxides – H_2O_2, cumene hydroperoxide and tert-butyl hydroperoxide – were tested against the KOTDR11 and KOTDR18 parasite populations (table 5.2). Because of the glutaredoxin-like *in vitro* activities of rTDR1, it was hypothesised that parasites lacking TDR1 may be more sensitive to these oxidising agents. However, the IC_{50} values obtained were broadly similar for each substance between the WT and TDR1 knockout parasites. IC_{50} values were also calculated for several compounds against WT, KOTDR11A, KOTDR11B and KOTDR18B parasites (table 5.3). The substances tested were the trivalent antimonial compound potassium antimonial tartrate (Sb(III)), which is toxic to *Leishmania* and affects the redox balance of the parasites (Wyllie *et al.*, 2004); juglone, which is toxic to trypanosomes (Akerman and Muller 2005) and produces intracellular ROS (Kampkotter *et al.*, 2003); N-methylphenazinium methyl sulphate, which is toxic to trypanosomes (Akerman and Muller 2005) and generates intracellular O_2^- (Maridonneau *et al.*, 1983) and β-mercaptoethanol, a chemical that reduces disulphide bonds and affects the expression levels of *E. coli* glutaredoxins (Potamitou *et al.*, 2002). Both the KOTDR11A and KOTDR11B lines were considerably more sensitive to paraquat (IC_{50} values of 1.2 and 1.4 mM, respectively) than WT parasites (IC_{50} value of 5.5 mM). Therefore the effect of paraquat on the different parasite lines was investigated further.

	WT	KOTDR11	KOTDR18
H_2O_2	234.5 ±10.7	328.7 ±29.9	227.8 ±13.8
Cumene hydroperoxide	9.4 ±1.2	9.4 ±1.3	9.0 ±0.6
Tert-butyl hydroperoxide	27.3 ±1.7	14.4 ±6.5	27.2 ±2.6

Table 5.2: IC_{50} values of H_2O_2, cumene hydroperoxide and tert-butyl hydroperoxide against WT, KOTDR11 and KOTDR18 *L. major* promastigotes. All values were obtained using the acid phosphatase assay. The values and standard deviations were calculated using the Grafit software IC_{50} programme. All values are in μM.

	WT	KOTDR11A	KOTDR11B	KOTDR18B
Sb(III)	23.2 ±2.7	16.7 ±3.0	N/D	N/D
β-me	330.8 ±21.9	301.4 ±36.3	N/D	N/D
juglone	2.3 ±0.7	2.4 ±0.3	N/D	N/D
H_2O_2	253.3 ±2.9	264.4 ±6.8	266.6 ±7.6	290.6 ±8.1
paraquat	5500 ±1004	1200 ±100	1400 ±500	N/D
NMPMS	10.1 ±0.4	7.8 ±0.54	N/D	N/D

Table 5.3: IC_{50} values of different compounds against WT, KOTDR11 and KOTDR18 *L. major* promastigotes. All values were obtained using the Alamar Blue assay. The values and standard deviations were calculated using the Grafit software IC_{50} programme. All values are in μM. Sb(III), potassium antimonial tartrate; β-me, β-mercaptoethanol; NMPMS, N-methylphenazinium methyl sulphate; N/D, not determined.

5.3.3.7 Effect of agents that induce oxidative stress on TDR1 expression in *L. major* promastigotes

In chapter four the varying expression of TDR1 between different life-cycle stages was demonstrated. Given that TDR1 displays thioltransferase and DHAR activities *in vitro*, it may have a role in protecting the parasite from oxidative stress and therefore TDR1 expression may be modulated by fluctuations in the intracellular redox environment of the parasite. In order to determine whether exposure to oxidative stress could stimulate TDR1 expression in WT *L. major* promastigotes, parasites were incubated for varying times with different concentrations of H_2O_2 or paraquat and western blots were performed on the resultant parasite lysates (figure 5.22). The protein concentrations of the samples were determined and normalised, but to ensure equal loading of the samples CS and/or transketolase (TK) expression was examined in parallel with TDR1, using the rabbit anti-CS and anti-TK antibodies. TDR1 expression did not change in *L. major* parasites incubated with 125 μM H_2O_2 at any of the time-points tested, nor in parasites incubated with 500 μM H_2O_2 after 4 hours (at later time-points the parasites were mostly dead so the TDR1 expression could not be determined) (figure 5.22A). It is possible any changes in expression would take place before the initial four hour time-point when the parasites were

harvested. Accordingly, *L major* parasites were incubated with paraquat for less time, with the parasites being harvested at three time-points within 24 hours of initial exposure to the oxidant. However, no variations in TDR1 expression were visible at any of these time-points with the different paraquat concentrations (figure 5.22B). TDR1 may have a role in protecting the parasite against oxidative stress; however, its expression is not affected by exposure to these oxidants under the conditions tested.

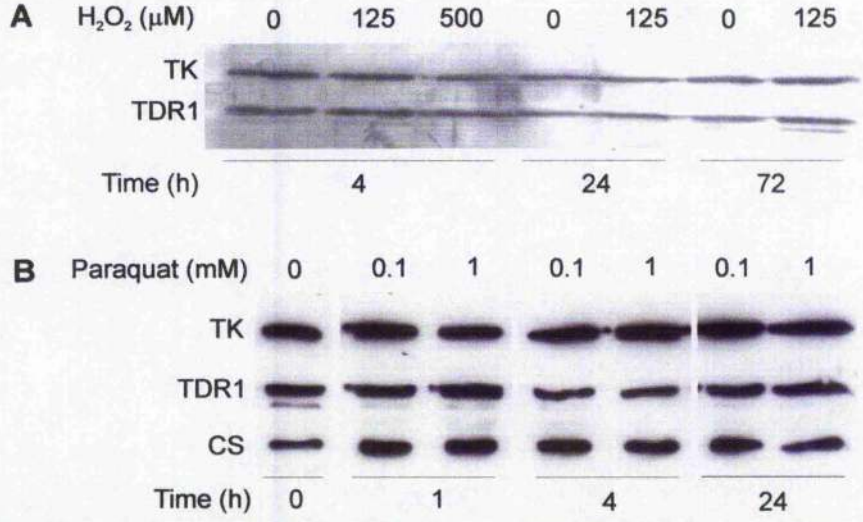

Figure 5.22: Western blot analysis of the effect of H_2O_2 and paraquat on TDR1 expression in *L. major* promastigotes. A – parasites inbubated with H_2O_2. 12 μg of soluble parasite lysate loaded per lane. B – parasites incubated with paraquat. 16 μg of soluble parasite lysate loaded per lane. The concentrations of the reagents are shown above each figure and the length of exposure time is shown below in h (hours). TK (transketolase) and CS (cysteine synthase) were used as loading control proteins. The identities of the different proteins are indicated to the left of the image. There was no noticeable change in TDR1 expression under the conditions tested.

5.3.3.8 Growth of promastigotes with paraquat

The IC_{50} data presented in section 5.3.3.6 showed that the TDR1 knockout lines KOTDR11A and KOTDR11B are approximately four times more sensitive to paraquat than WT parasites. To further investigate the effect of paraquat on these lines, WT, KOTDR11A, KOTDR11B and KOTDR18 parasite cultures were seeded at 1 x 10^6/ml and grown with 5 mM paraquat, and the growth of the parasites' was monitored by counting (figure 5.23). While the parasite density of the WT culture increased to approximately 2 x 10^7 after six days, over the same time period the parasite density of the three TDR1 knockout lines fell to 1.4-2.6 x 10^5. This result reflects the IC_{50} data presented previously

and demonstrates a clear difference in the sensitivity of the TDR1 knockout lines to paraquat as compared to WT parasites.

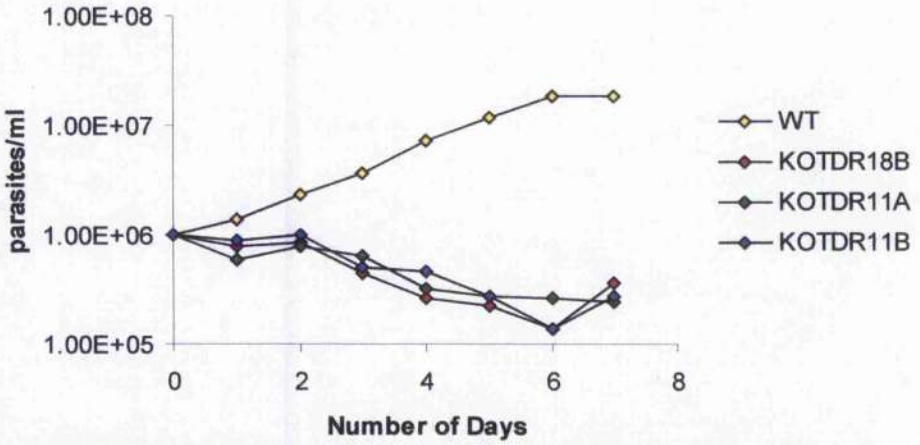

Figure 5.23: Effect of paraquat on the growth of WT, KOTDR18B, KOTDR11A and KOTDR11B *L. major* promastigotes. The parasites were seeded at 1 x 10^6 with 5 mM paraquat and were counted daily.

5.4 Re-expressing TDR1 in TDR1 knock-out parasite lines

In order to clarify whether any phenotype observed in the TDR1 knockout lines was due to the loss of TDR1 as opposed to any secondary mutation or damage the parasites may have accumulated, TDR1 was re-expressed in the knockout lines. This was achieved by transforming the KOTDR11A, KOTDR11B and KOTDR18 parasites with the pGL102TDR1 plasmid used to over-express TDR1 in WT *L. major*, as described in section 5.2.1. In addition the same lines were transformed with the pGL102 plasmid so they could be used as negative control samples. These plasmids were previously presented in figure 5.1. Using the names of the original lines and plasmids used to transform them, the new lines were named KOTDR11A + pGL102, KOTDR11A + pGL102TDR1, KOTDR11B + pGL102, KOTDR11B + pGL102TDR1, KOTDR18B + pGL102 and KOTDR18B + pGL102TDR1.

5.4.1 Analysis of re-expression

Whether the TDR1 knockout parasites transformed with pGL102TDR1 that grew in the presence of the selective drug had been successfully transformed, resulting in TDR1 being re-expressed, was assessed by western blot analysis. WT, KOTDR11A + pGL102TDR1, KOTDR11B + pGL102TDR1 and KOTDR18B + pGL102TDR1 promastigotes grown with neomycin were harvested and lysed, and the soluble fractions were separated by SDS-PAGE before western blotting. The sheep ani-TDR1 antibody was used to detect TDR1 expression. As a positive control, CS expression was examined in parallel with TDR1, using the rabbit anti-CS antibody as before. TDR1 was expressed in all three re-expressing lines (figure 5.24)

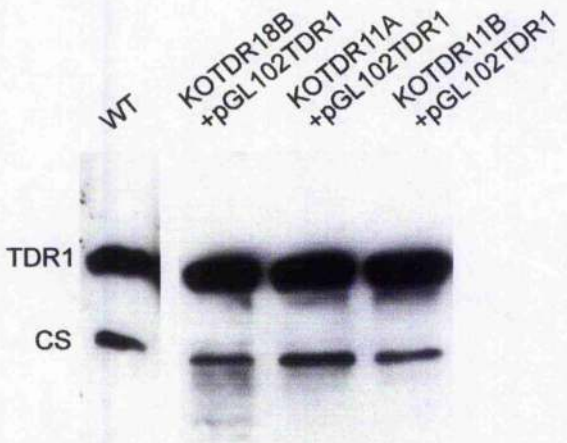

Figure 5.24: Western blot analysis of TDR1 expression in pGL102 *L. major* promastigotes and KOTDR1 *L. major* promastigotes transformed with pGL102TDR1. 10-20 µg of soluble parasite lysate was loaded per lane. CS, cysteine synthase protein, was used as a positive control. The identities of the different proteins are indicated to the left of the image. TDR1 was re-expressed in each of the lines tested.

5.4.2 Growth of parasites re-expressing TDR1, with paraquat

The IC_{50} data presented in section 5.3.3.6, together with the growth curves presented in section 5.3.3.8, show that the TDR1 knockout lines KOTDR11A and KOTDR11B are more sensitive to paraquat than WT parasites. To investigate whether the loss of TDR1 was responsible for this phenotype, the parasite lines re-expressing TDR1 were grown with paraquat. WT, KOTDR11A, KOTDR11B, KOTDR11A + pGL102, KOTDR11A + pGL102TDR1, KOTDR11B + pGL102, KOTDR11B + pGL102TDR1 parasite cultures were seeded at 1 x 10^6/ml and grown with 5 mM paraquat, and growth was monitored by

counting (figure 5.25). While the parasite density of the WT culture increased as before, the density of all the other lines decreased in a similar rate to the TDR1 knockout parasites. Clearly, the re-expression of TDR1, which was confirmed by western blot analysis, does not complement the paraquat-sensitive phenotype of the TDR1 knockout cultures. Whether this is due to the original phenotype being caused by a factor other than the loss of TDR1, or whether TDR1 re-expression does not reinstate the parasites' relative insensitivity to paraquat for some other reason, is currently unknown.

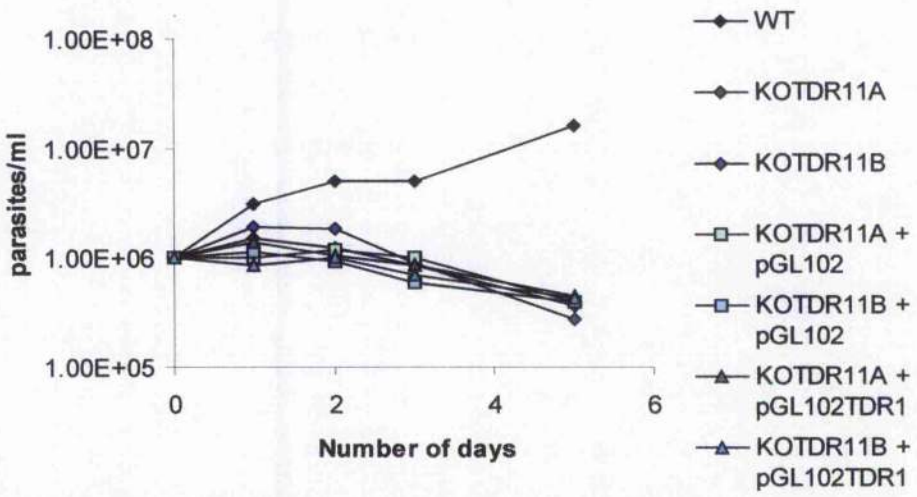

Figure 5.25: Effect of paraquat on the growth of WT *L. major* promastigotes and KOTDR1 *L. major* promastigotes transformed with pGL102 or pGL102TDR1. The parasites were seeded at 1 x 10^6/ml with 5 mM paraquat and were counted daily. With the exception of WT, KOTDR11A and KOTDR11B, the parasites were also grown with 50 µg/ml neomycin.

5.5 Discussion

The *in vitro* characteristics of rTDR1 described in the introduction to this chapter are consistent with the observed activities of oGST. rTDR1 exhibited GSH-CDNB conjugating activity which is characteristic of other types of GSTs, but as for human oGST, it was at a very low level. However, this activity should not be ignored as although an elongation 1B complex displaying trypanothione S-transferase activities was recently identified in *L. major* (Vickers *et al.*, 2004), no known GSTs have ever been found in *Leishmania*. Therefore TDR1 is the first protein with GST activity to be identified in the parasite.

However, the more notable functions displayed by rTDR1 were DHAR and thioltransferase activities. Whether the DHAR activity displayed by TDR1 is of relevance is an interesting question. Although ascorbate has been detected in *T. cruzi* (Clark *et al.*, 1994), it has never been detected in *Leishmania* although an ascorbate-dependant peroxidase has also been identified (Adak and Datta, 2005). However, trypanothione is able to reduce dehydroascorbate (Krauth-Siegel and Ludemann, 1996) and may be sufficient to maintain ascorbate levels without the need for an enzymatic reductase. The issue of ascorbate being necessary in *Leishmania*, and therefore whether TDR1 may have a role in maintaining it in the reduced from, is addressed in chapter six. The high thioltransferase activity exhibited by TDR1 is the only *in vitro* enzymatic capability that was also displayed by the most closely related protein to TDR1, Tc52. CDNB-GSH conjugating activity was not detected in the *T. cruzi* protein (Moutiez *et al.*, 1995) and it was not assessed for DHAR (or indeed metal-reducing) activities. It is possible that the active site differences between TDR1 and Tc52 described in chapter three account for these differences. As mentioned, DHAR and thioltransferase activities are commonly exhibited by glutaredoxins, a class of proteins that share predicted active site sequences with TDR1 but are otherwise much smaller and rather dissimilar. As described in chapter three, *L. major* contains several proteins that are annotated as, and share sequence similarity to, glutaredoxins. Organisms often possess several GRXs that may display redundancy towards each other due to their overlapping functions (Draculic *et al.*, 2000). Therefore the possibility of redundancy between TDR1 and other *Leishmania* proteins must be taken into account when considering the glutaredoxin-like role of TDR1 *in vivo*.

To investigate the role of TDR1 *in vivo*, parasites over-expressing TDR1 (figure 5.2) and clonal TDR1 knockout lines (figure 5.9) were created. The growth rates of all these lines were assessed and were found to be similar to that of WT promastigotes (figures 5.3 and 5.10) with the exception of one of knockout lines, KOTDR18A. The growth rate of this

line, together with the parasite density in stationary-phase cultures, was slightly impaired and analysis of the line was discontinued. The metacyclogenesis of the TDR1 knockout lines was assessed and was shown to be broadly similar to that of WT parasites in all the lines tested. These findings contrast with the observations of *T. cruzi* parasites that had reduced levels of Tc52: epimastigotes did not grow well in culture and were impaired in their ability to differentiate into metacyclic forms (Allaoui *et al.*, 1999). Moreover, only a mono-allelic Tc52 knockout line was obtained; knocking out both copies of Tc52 is thought to be lethal.

The IC_{50} values of several compounds against WT TDR1 knockout, and TDR1 over-expressing lines were calculated in order to determine if the level of TDR1 affected the sensitivity of parasites to different types of stress (tables 5.1, 5.2 and 5.3). There was no consistent significant difference in the IC_{50} values of most of the compounds tested between the different lines, including the hydroperoxides, potassium antimonial tartrate and β-mercaptoethanol. However, several agents that induce the production of intracellular ROS were tested and a significant difference in the IC_{50} value of one of these – paraquat – was apparent between WT parasites and those lacking TDR1. This finding was reinforced by the observation that WT cells were much more tolerant to paraquat than TDR1 knockout parasites were, when the lines were grown in the presence of the chemical (figure 5.23).

Glutaredoxins (GRXs) have been previously linked with protecting cells against paraquat-induced oxidative stress. Knocking out the WT and expressing a mutated version of GRX5 in *Schizosaccharomyces pombe* resulted in increased sensitivity to paraquat (Chung *et al.*, 2005) and a similar phenotype was observed in *S. pombe* GRX2 null mutants (Chung *et al.*, 2004). These reports reflect the increased sensitivity of TDR1 knockout parasites to paraquat that are described in this chapter, and support the hypothesis based on the *in vitro* activities of the protein that TDR1 functions as a glutaredoxin in *Leishmania*.

A possible reason for the increase in paraquat sensitivity of the GRX mutants could be that they have depleted ascorbate levels (due to a lack of DHAR activity). Ascorbate is a low-molecular weight antioxidant which can react directly with ROS, and is the essential co-factor for ascorbate-dependant peroxidases, proteins that enzymatically reduce H_2O_2. Paraquat treatment caused dehydroascorbate reductase activity to increase in *Conyza bonariensis* plants (Ye and Gressel, 2000) and TRX1 levels to increase in mouse liver and lung tissues (Jurado *et al.*, 2003). Although the expression of TDR1 in *L. major* was not affected by exposure to sub-lethal concentrations of paraquat (figure 5.22), this was also

found to be the case elsewhere: although exposure to the chemical did not result in increased GRX2 levels in *S. pombe* (Chung *et al.*, 2004), or GRX1 or GRX2 levels in mouse liver or lung tissues (Jurado *et al.*, 2003). Meanwhile, the expression of a human DHAR gene in tobacco plants caused them to be less susceptible to paraquat-induced damage (Kwon *et al.*, 2003). Over-expression of TDR1 did not cause the parasites to become more tolerant to the compound (table 5.1 and figure 5.7). This would be the expected result if ascorbate recycling is not the rate-limiting step in ROS detoxification in *Leishmania*.

If the loss of TDR1 is responsible for the increased sensitivity to paraquat of the TDR1 knockout lines, re-expressing TDR1 in these lines should restore resistance to the chemical as displayed by WT parasites. Despite confirmation that TDR1 was being re-expressed by parasites transformed with pGL102TDR1, the susceptibility of these lines to paraquat did not decrease (figure 5.25). There are several possible reasons for this. It may be the case that some other mutation or damage to the TDR1 knockout lines is responsible for their increased sensitivity to paraquat. However, this seems unlikely given that all three independently derived knockout lines exhibit the same phenotype. Another possibility is that an aspect of the TDR1 re-expression does not comply with WT expression and that this effectively inactivates the protein. For example, the expression level or the localisation of TDR1 may differ in the re-expressing lines. Further analysis is required to determine which of these scenarios is correct. Re-expressing TDR1 from the original gene locus or from another chromosomal location may also be a helpful approach.

L. major parasites over-expressing TDR1 were more infective to macrophages than WT and pGL102 control parasites (figure 5.4), and were also more infective to mice than the control line (figure 5.5). Paradoxically, the TDR1 knockout lines were also more infective to macrophages (section 5.3.3.3) and, to a lesser extent, mice, than WT parasites (section 5.3.3.4). It was also notable that the percentage of macrophages with WT parasites appeared to be more susceptible to a prolonged incubation time and an increase in incubation temperature than the TDR1 knockout parasites (figures 5.13-5.15). The reasons for the apparent contradictions between the TDR1 over-expressing and knockout infection data are unclear. The pGL102 line which acted as a control for the TDR1 over-expressing line appears to be in some way damaged: the parasites were less infective than WT parasites to both macrophages and mice. However, misinterpretation of the TDR1 over-expressing line infection data due to it being compared to a faulty control is not to blame, as the TDR1 over-expressing line was also more infective than WT. Whether the WT line itself could also be impaired was considered. However, the utilisation of a second WT line

which was of a different passage number gave very similar macrophage infection data to the original WT line (figures 5.14 and 5.15), suggesting that this was not the case. One possibility is that when TDR1 is knocked out, the levels of other proteins – possibly the glutaredoxin-like proteins described in chapter three – are modulated to compensate for the loss. This in turn could have a positive effect on the TDR1 knockout parasites, improving their viability *in vivo*. Meanwhile, given the glutaredoxin-like properties of TDR1, over-expression of the protein could have a more straightforward beneficial effect on *L. major*; the increased infectivity may be due to them being better equipped to cope with the oxidative stress encountered upon invasion of a cell.

Once again, the results do not correspond with the Tc52 knockout and over-expresser data. Although there was no difference between the infectivity of WT *T. cruzi* and parasites over-expressing Tc52, parasites with decreased Tc52 levels were significantly less infective to both macrophages and mice than WT parasites (Allaoui *et al.*, 1999). Moreover Tc52 knockout parasites that do infect animals result in an attenuated form of Chagas' disease with reduced parasitemia compared to WT infections (Garzon *et al.*, 2003). Tc52 is secreted and is thought to have a role in modulating the immune response of the host (Fernandez-Gomez *et al.*, 1998; Borges *et al.*, 2001; Ouaissi *et al.*, 2002; Garzon *et al.*, 2003), which is hypothesised to be the reason behind the reduced infectivity displayed by parasites with decreased levels of Tc52 (Garzon *et al.*, 2003). It was shown in chapter four that TDR1 is also released from promastigote parasites but the increased infectivity of parasites lacking TDR1 suggests that the protein does not perform the same function as Tc52.

As described in the introduction to this chapter, rTDR1 was able to reduce pentavalent antimonials to the more toxic trivalent forms *in vitro*. This was in keeping with the ability of mammalian oGST to reduce pentavalent arsenicals (Zakharyan *et al.*, 2001), and supported the hypothesis that TDR1 may be involved in mediating antimonial susceptibility in *Leishmania*. To resolve whether TDR1 did indeed perform this function in vivo, macrophages infected with WT, TDR1 over-expressing and TDR1 knockout *L. major* parasites were incubated with sodium stibogluconate (Sb(V)) and the impact of the drug on the infections was assessed. The effect of Sb(V) on *L. major* promastigotes could not be tested as they are not sensitive to the trivalent drug. However, it proved to be extremely difficult to assess the impact of Sb(V) on *L. major* macrophage infections for two main reasons: the difference in the basal macrophage infection rates of the various lines, and the insensitivity of all the lines tested to the drug.

There appeared to be no significant difference between the susceptibility of TDR1 over-expressing and control parasites to the drug (figure 5.6). When considering the raw data on the effect of Sb(V) on the macrophage infections of the WT and TDR1 knockout, it looks like the knockout parasites are highly insensitive to the drug, consistent with the idea that TDR1 mediates antimonial toxicity. Even at the highest concentrations of Sb(V), approximately 30-40% of macrophages were infected with the TDR1 knockout parasites, compared to only 5% infected with WT parasites (figure 5.19). Similar results were achieved in other experiments (section 5.3.3.5). However, due to the different infectivity of WT and TDR1 knockout parasites to macrophages when no drug was present, in order to directly compare the effect of Sb(V) between the lines, it was necessary to consider the effect of the drug on the reduction of the initial infection level. Therefore, the data for the infection levels achieved with the varying drug concentrations was converted to the percentage of the infection levels achieved with no drug was present. This makes the data look very different and shows that despite the impressive infection levels of the knockout lines achieved with high concentrations of the drugs, the reduction in the infection level is similar to that of the WT parasites. However, manipulating the data in this way is not an ideal solution; similar basal infection levels between the lines would allow a proper comparison of the effect of Sb(V). A useful approach may be to analyse the effect of Sb(V) on *in vivo* infections of the different lines, as these were at a more similar basal level than the macrophage infections. This would also circumvent the problem of the apparent insensitivity of *L. major* parasites in macrophages to Sb(V).

While this study was in progress, another arsenate reductase-like protein that could reduce pentavalent metalloids *in vitro* was identified, LmACR2 (Zhou *et al.*, 2004). Although LmACR2 knockout parasites were not constructed, the protein was over-expressed in L. *infantum* and this resulted in an increase in sensitivity to Sb(V). The effect of Sb(V) was assessed using a similar macrophage infection assay to the one used in this investigation. The approximate Sb(V) IC_{50} value, as calculated from their findings, was 120 μg/ml against parasites over-expressing LmACR2, and 180 μg/ml against WT parasites. Clearly this is a much lower value than in the experiments detailed in this chapter. Although there was variation between experiments and the incubation temperature affected the Sb(V) efficacy, the IC50 was approximately 500-1000 μg/ml, a concentration that also appeared to be toxic to the macrophages. Therefore, manipulating the expression of TDR1 in a *Leishmania* strain that is more susceptible to Sb(V) may help to ascertain whether the protein does indeed mediated antimonial sensitivity *in vivo*.

The TDR1 over-expressing and knockout *L. major* lines generated were useful tools in analysing the role of TDR1 *in vivo*. The unexpected ease in constructing null mutants – which could not be achieved for Tc52 in *T. cruzi* – together with the observations that the resultant parasites proliferated well *in vitro* and were infectious to aimals and cells showed that TDR1 was non-essential in *L. major*. The protein is likely to function similar to a glutaredoxin and may protect parasites from oxidative stress. The fact that *L. major* contains several glutaredoxin-like sequences and that redundancy is known to occur between glutaredoxins may explain why the parasites were able to lose TDR1 so easily. Another reason could be that the protein is only essential when the parasites are residing in the sandfly vector. Whether TDR1 affects the *Leishmania* susceptibility to antimonials remains unresolved. A recent study has shown that the expression of TDR1 was not modulated in antimonial resistant field-isolates (Decuypere *et al.*, 2005), suggesting that the protein is not responsible for drug-resistance. However, in this study protein expression was interpreted from the RNA transcript levels; because DNA transcription in *Leishmania* is polycistronic the relevance of this observation is unclear. However, in order to determine whether the protein is able to reduce the pentavalent dugs *in vivo*, further experimentation is required. Analysis of the effect of Sb(V) on animals infected with the different lines generated here may help to clarify the impact of the protein.

6 Functional study of L-gulono lactone oxidase in *L. major*

6.1 Introduction

6.1.1 The role of ascorbate in the cell

Like GSH, ascorbate – commonly known as vitamin C – is a low-molecular weight antioxidant which can react directly with ROS, donating an electron which stabilises the free radical (Rose and Bode, 1993). In turn the ascorbate is oxidised and forms monodehydroascorbate which can then be recycled back to the reduced form – directly or via dehydroascorbate by enzymes that display dehydroascorbate reductase activity – as discussed in section 6.1.4. The highly reactive hydroxyl radical, which is not thought to be detoxified enzymatically, can be neutralised in this way. Ascorbate is present in high concentrations in many types of both mammal (Rose and Bode, 1993) and plant (Agius *et al.,* 2003; Chen *et al.,* 2003) tissues and is therefore an important antioxidant. As well as participating in reactions with free radicals directly, ascorbate has a further role in counteracting oxidative stress by acting as the electron donor in reactions mediated by ascorbate-dependant peroxidases (APXs) which are involved in the detoxification of H_2O_2. While the antioxidant enzyme catalase, which has a high turnover rate, is important in removing H_2O_2, it has a low affinity for the compound and is not suitable for managing low concentrations of it. On the contrary, peroxidases – including APXs – have a high affinity for H_2O_2 and can scavenge it even at low concentrations. The control of H_2O_2 levels is of the utmost importance, given its emerging role in the control of signal transduction and gene expression (Noctor *et al.,* 2000). Many APX isoenzymes with differing sub-cellular localisations are known to exist (reviewed in Shigeoka *et al.,* 2002) and have been especially well characterised in plants. However APX has also been found in bovine eye tissue (Wada *et al.,* 1998) and several types of eukaryotic algae including *Euglena gracilis* (Ishikawa *et al.,* 1996) which, like the trypanosomatids, lacks catalase. The absence of catalase in these organisms is interesting as it may be indicative of peroxidases having even greater importance in the elimination of oxidative stress. As discussed in section 6.1.5, APX has also been found in both *T. cruzi* and *L. major*.

Ascorbate has a clear role in protecting cells from oxidative stress and this is demonstrated by the phenotypes of various mutants of different organisms that have abnormal levels of ascorbate. The lack of reports of plant lines that are completely deficient in ascorbate

suggests that it is an essential compound in plants. However, various *A. thaliana* lines which have low levels of ascorbate – deemed vtc mutants – are hypersensitive to ozone (Conklin *et al.,* 2000) and salt stress (Huang *et al.,* 2005), and have growth deficiencies (Pavet *et al.,* 2005). Plants engineered to over-express an ascorbate peroxidase were less sensitive to various types of oxidative stress (Murgia *et al.,* 2004). Meanwhile *S. cerevisiae* engineered to lack D-arabinono-1,4-lactone oxidase (ALO) – one of the proteins required for ascorbate biosynthesis in yeast – exhibited increased sensitivity to both H_2O_2 and menadione, while yeast over-expressing the protein were less sensitive to these oxidants (Huh *et al.,* 1998). Ascorbate synthesis was enhanced in rat cells treated with oxidative-stress-inducing agents; when this was prevented by simultaneous treatment with sorbinil (which inhibits ascorbate synthesis) greater levels of ROS were found in the cells (Chan *et al.,* 2005).

Although ascorbate has an unequivocal role as an antioxidant, it is also involved in many other cellular processes (reviewed in Arrigoni and De Tullio, 2002; De Tullio and Arrigoni, 2004). These include synthesis of various cell compounds and cell-wall synthesis (Smirnoff, 2000), cell division (Liso *et al.,* 1984) and elongation (Smirnoff and Wheeler, 2000). The part ascorbate plays in many of these processes is explained by its role in reactions mediated by dioxygenases, a large group of enzymes that catalyse the incorporation of O_2 into an organic substrate and are themselves responsible for a wide range of reactions. Ascorbate-dependant dioxygenases (AADs) are a sub-group of these proteins which can use ascorbate as a co-substrate: indeed some specifically require it (Dong *et al.,* 1992). An important reaction catalysed by an AAD called P4H is the hydroxylation at carbon 4 of proline residues to be incorporated into polypeptide chains such as collagen and the transcription factor HIFα (Myllyharju, 2003). Depletion of ascorbate affects proline hydroxylation in guinea pigs and results in reduced collagen synthesis, in turn leading to scurvy-like symptoms (Peterkofsky, 1991). In tobacco plants, inhibition of P4H alters cell-wall synthesis and cell division (Cooper *et al.,* 1994). Other AADs include N-trimethyl-L-lysine hydroxylase and gamma-buytro-betaine hydroxylase which together are essential for the synthesis of carnitine, an amino acid derivative involved in fatty acid metabolism. Carnitine synthesis is affected by ascorbate levels (Ha *et al.,* 1991) and its depletion is responsible for several phenotypes including muscle weakness in humans (http://www.emedicine.com/PED/topic321.htm). AADs are also implicated in hormone and flavonoid synthesis in plants (Arrigoni and De Tullio, 2002) and in the control of the cell cycle (De Tullio and Arrigoni, 2004). Although this account of the processes ascorbate is involved in is not exhaustive, the diverse range of activities dependant on ascorbate and its resultant significance in the cell is clear.

6.1.2 Ascorbate synthesis

Ascorbate or the similar compound erythroascorbate are produced in a wide variety of eukaryotic organisms albeit by several different biosynthetic pathways (see figure 6.1). In most mammals ascorbate is synthesised from glucose via the uronic acid pathway, first proposed in the 1950s (Isherwood *et al.,* 1954). Interestingly humans (along with other primates and guinea pigs) lack the L-gulonolactone oxidase enzyme required for the final step in ascorbate synthesis (Sato and Udenfriend, 1978). Instead, they obtain ascorbate from plants, which contain high levels of ascorbate. Indeed plants employ several different biosynthetic pathways to convert D-glucose-6-Phosphate, D-fructose-6-phosphate or D-mannose-6-phosphate to ascorbate (Wheeler *et al.,* 1998; Valpuesta and Botella, 2004). Meanwhile, some fungi and yeast species synthesise D-erythroascorbate rather than ascorbate (Smirnoff, 2001). These two compounds are very similar and are thought to have similar functions within the cell; indeed the characterised enzymes that mediate D-erythroascorbate synthesis are similar to enzymes involved in ascorbate synthesis from other organisms. The various known ascorbate (and D-erythroascorbate) synthesis pathways are diagrammatically shown in figure 6.1, and characterised enzymes known to be involved in ascorbate synthesis are discussed below and summarised in table 6.1.

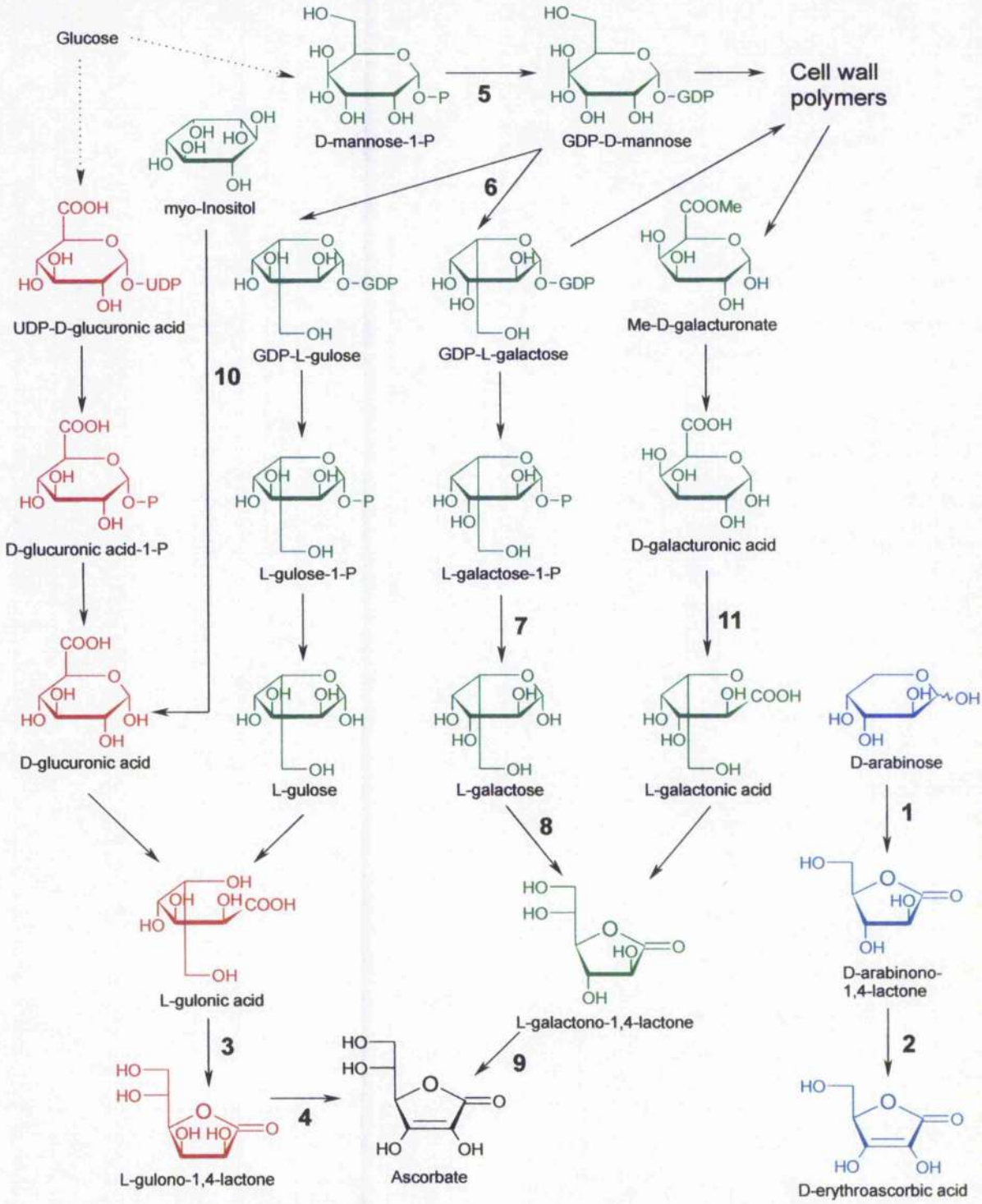

Figure 6.1: The biosynthesis of ascorbate in mammals, plants and fungi. The proposed mammalian synthesis pathway is shown in red, the proposed plant pathways are shown in green and the proposed fungi pathway is shown in blue. The steps in the pathways are numbered where an enzyme able to catalyse the reaction has been identified and the numbers correspond with those in tables 6.1 and 6.2. 1, D-arabinose dehydrogenase; 2, D-arabinono-1,4-lactone oxidase (ALO); 3, Aldono-lactonase; 4. L-gulonolactone oxidase (GLO); 5, GDP-mannose pyrophosphorylase; 6, GDP-mannose-3',5'-epimerase; 7, L-galactose-1-phosphate phosphatase; 8, L-galactose dehydrogenase; 9, L-galactono-1,4-lactone dehydrogenase (GALDH); 10, Inositol oxygenase; 11, D-galacturonic reductase.

6.1.2.1 Biosynthesis in mammals

Although the mammalian ascorbate synthesis pathway is well established, only two of the enzymes involved have been purified and characterised to date: a very recently identified aldono-lactonase, thought to catalyse the formation of L-gulono-1,4-lactone from L-gulonic acid, and L-gulonolactone oxidase (GLO), which catalyses the formation of ascorbate from L-gulono-1,4-lactone. These are the penultimate and final reactions in ascorbate biosynthesis, respectively. It has been suggested that the recently identified mouse aldono-lactonase is responsible for L-gulono-1,4-lactone formation (Kondo *et al.*, 2006). However only the reverse reaction – hydrolysis of L-gulono-1,4-lactone – has been detected and characterised. However, mice lacking this enzyme and fed an ascorbate-free diet developed symptoms of scurvy and had greatly reduced ascorbate levels compared to wild-type mice, showing that the protein is critical for normal ascorbate production. GLO, which is absent in humans (the reason for susceptibility to scurvy), is expressed primarily in liver and kidneys and is localised to the microsomes of cells (Kiuchi *et al.*, 1982; Puskas *et al.*, 1998). Naturally occurring disruptions of the gene that encodes GLO in both pigs and mouse cause ascorbate deficiency (Hasan *et al.*, 2004; Mohan *et al.*, 2005). Surprisingly few reports have been recently published on this protein: there is no account of a gene encoding mammalian L-gulonolactone oxidase being cloned.

6.1.2.2 Biosynthesis in plants

The production of ascorbate in plants is complicated by the apparent existence of several different biosynthesis pathways (Valpuesta and Botella, 2004) that have not yet been firmly established. Currently three different pathways are proposed to exist (see figure 6.1): the first, with L-galactose as an intermediate; the second, where ascorbate is synthesised from *myo*-inositol; and thirdly with galacturonic acid as a precursor.

The L-galactose pathway is the most extensively characterised pathway of the three and most steps have been experimentally confirmed (Wheeler *et al.*, 1998). *Arabidopsis thaliana* leaves were shown to synthesise ascorbate from both glucose and mannose (Wheeler *et al.*, 1998), although a higher percentage of mannose is converted to ascorbate as it participates in fewer additional cellular reactions. It is well established that GDP-D-mannose and GDP-L-galactose are synthesised from D-glucose-6-phosphate via several intermediates for participation in polysaccharide synthesis (Smirnoff, 2000). L-galactose is thought to be formed by a two-step hydrolysis of GDP-L-galactose, the second step of which has been recently found to be catalysed in kiwifruit and *A. thaliana* by L-galactose-

1-phosphate phosphatase (Laing *et al.,* 2004). Thereafter L-galactose is converted to L-galactono-1,4-lactone by L-galactose dehydrogenase (Gatzek *et al.,* 2002) before ascorbate is finally produced after catalysis by the mitochondrial L-galactono-1,4-lactone dehydrogenase (GALDH) (Ostergaard *et al.,* 1997). GALDH performs an analogous function to, and shares amino acid sequence similarity with, mammalian GLO: cauliflower GALDH shares 27% amino acid identity with GLO from rat, although GALDH has a large N-terminal extension relative to GLO. Meanwhile L-galactose dehydrogenase is not particularly similar to mouse aldono-lactonase, the enzyme thought to catalyse the penultimate step in ascorbate production in mammals: they share just 10% identity at the amino acid level. The L-galactose pathway has been further complicated by the finding that GDP-mannose-3',5'-epimerase can catalyse the formation of GDP-L-gulose as well as GDP-L-galactose (Wolucka and Van Montagu, 2003). It has also been proposed that L-gulonic acid could be formed from GDP-L-gulose, and that this is converted to ascorbate via the same pathway that occurs in mammals (Valpuesta and Botella, 2004). This will be discussed in more detail in relation to the *myo*-inositol pathway below.

The conversion of GDP-L-galactose to L-galactose is considered to be the first reaction in the pathway specific to ascorbate synthesis (Wheeler *et al.,* 1998). However, both GDP-mannose pyrophosphorylase and GDP-mannose-3',5'-epimerase, which catalyse the previous two steps in the pathway, have been postulated to have an important role in ascorbate synthesis as they are inhibited and down-regulated, respectively, upon addition of exogenous ascorbate *in vivo* (Tabata *et al.,* 2002; Wolucka and Van Montagu, 2003). Furthermore, mutations in *A. thaliana* lines defective in ascorbate synthesis mapped to the GDP-mannose pyrophosphorylase gene (Conklin *et al.,* 1999). Evidence also exists for both L-galactose dehydrogenase and GALDH directly affecting *in vivo* ascorbate levels: ascorbate levels decrease when antisense L-galactose dehydrogenase (Gatzek *et al.,* 2002) or GALDH (Tabata *et al.,* 2001) RNA transcripts are expressed in *A. thaliana*.

The formation of ascorbate from *myo*-inositol is a less well-characterised route of biosynthesis. It was previously known that plants can derive *myo*-inositol from glucose-6-phosphate (Naccarato *et al.,* 1974) and that *myo*-inositol could be in turn converted to D-glucuronic acid by inositol oxygenase (Reddy *et al.,* 1981). Recently this enzyme was found in *A. thaliana* and when over-expressed was shown to significantly increase the amount of ascorbate in the plant (Lorence *et al.,* 2004). Because D-glucuronic acid is an intermediate in the ascorbate biosynthesis pathway found in mammals (see figure 6.1), there has been speculation that the mammalian pathway may be operating in plants, albeit mediated by different enzymes. Indeed, over-expression of rat GLO in *A. thaliana* plants

deficient in ascorbate synthesis complemented the mutant phenotype (Radzio *et al.*, 2003). This is in accordance with the hypothesis that L-gulose, formed from GDP-D-mannose due to GDP-mannose-3',5'-epimerase activity (see above), can be converted to L-gulonic acid and then to ascorbate via the mammalian pathway. There is evidence to support the idea that the mammalian ascorbate biosynthetic pathway is operating in plants: when L-gulose and D-glucuronic acid were administered to *A. thaliana* cell culture, the rate of ascorbate synthesis increased (Davey *et al.*, 1999). In addition, L-gulonic acid has been detected in plants (Wagner *et al.*, 2003), and GLO activity has been detected in *A. thaliana* (Wolucka and Van Montagu, 2003). Clearly additional investigation is required to clarify whether part or the entire ascorbate biosynthetic pathway is also present in plants.

The third putative pathway of ascorbate synthesis in plants is from D-galacturonic acid, which is released in plants upon hydrolysis of cell wall breakdown products. It had been previously shown that pea extracts could catalyse the formation of ascorbate from methyl-D-galacuronate and galacturonic acid (Mapson and Isherwood, 1954) and more recently ascorbate synthesis was shown to be increased when these compounds were added to *A. thaliana* cell culture (Davey *et al.*, 1999). Enzymatic confirmation of this third pathway was provided when a D-galacturonic reductase cloned from strawberry was shown to convert D-galacturonic to L-galactonic acid, which is spontaneously converted to L-galactono-1,4-lactone, the substrate for GALDH (which is also synthesised in the L-galactose pathway) (Agius *et al.*, 2003). Moreover, over-expression of the *A. thaliana* version of the gene resulted in a significant increase in ascorbate levels within the plants. The authors of this paper have also suggested that the various pathways responsible for ascorbate synthesis within the cell may be developmentally regulated (Agius *et al.*, 2003), perhaps providing in part an explanation for the presence of more than one ascorbate biosynthetic pathway in the organism: perhaps different pathways operate at different points in the plant life-cycle or in different tissue-specific localisations.

6.1.2.3 Biosynthesis in yeast

As mentioned, yeast and other lower eukaryotes have been shown to synthesise the ascorbate analogue D-erythroascorbate from D-arabinose (see figure 6.1). However, when given the alternative substrates L-galactono-1,4-lactone and L-gulono-1,4-lactone (intermediate compounds from the plant and mammalian ascorbate biosynthetic pathways respectively), *S. cerevisiae* could also produce ascorbate, although L-gulono-1,4-lactone was a poor substrate (Spickett *et al.*, 2000). These observations show that the enzymes that mediate the pathway can use various substrates, either because they have evolved from a

common ancestor to similar proteins in plants and mammals, or because ascorbate or erythroascorbate are synthesised from whatever substrates are available.

D-arabinose is first converted to D-arabinono-1,4-lactone, possibly via D-arabinono-1,5-lactone, by D-arabinose dehydrogenase (ARA1), which has been purified and characterised from several organisms: *Candida albicans* (Kim *et al.*, 1996), *Neurospora crassa* (Carrasco *et al.*, 1981) and *S. cerevisiae* (Kim *et al.*, 1998). The *C. albicans* and *S. cerevisiae* enzymes can also use L-galactose as substrates, showing that this step in the biosynthetic pathway is similar to that in plants. However, when compared to the analogous *A. thaliana* L-galactose dehydrogenase enzyme, the amino acid sequences were just 17% identical. Moreover, *S. cerevisiae* ARA1 shares just 11% identity with the recently identified mouse aldono-lactonase. *S. cerevisiae* ARA1 is more similar to glycerol dehydrogenases and is 38% identical to glycerol dehydrogenase from *Aspergillus nidulans*. Whether plant L-galactose dehydrogenase displays similar substrate specificities as the fungal enzymes and can convert D-arabinose has not yet been investigated.

The final step in D-erythroascorbate synthesis is the conversion of D-arabinono-1,4-lactone, a reaction catalysed by D-arabinono-1,4-lactone oxidase (ALO). This has been purified and characterised from *S. cerevisiae* (Huh *et al.*, 1998) and *C. albicans* (Huh *et al.*, 1994). Both reported ALO proteins can catalyse the production of ascorbate from L-gulono-1,4-lactone and L-galactono-1,4-lactone (the penultimate compounds in the mammalian and plant ascorbate biosynthesis pathways, respectively), as well as D-erythroascorbate from D-arabinono-1,4-lactone. Indeed, ALO is similar to GLO and GALDH: the amino acid sequence of *S. cerevisiae* ALO shares 32% and 21% identity with those of rat GLO and cauliflower GALDH, respectively (Huh *et al.*, 1998). Therefore, at least at the amino acid level, ALO is significantly more similar to mammalian GLO than to plant GALDH. This is perhaps surprising given that yeast ARA1 is more similar to *A. thaliana* L-galactose dehydrogenase than to the mouse aldono-lactonase thought to catalyse the penultimate reaction in ascorbate biosynthesis. Elucidation of which substrates these plant and mammalian enzymes are capable of acting on will clarify which pathway D-erythroascorbate synthesis resembles more.

Enzyme Name	Organisms found in	Reaction catalysed
1. D-arabinose dehydrogenase (ARA1)	Yeast and fungi: *N. crassa, C. albicans, S. cerevisiae*	D-arabinose to D-arabinono-1,4-lactone
2. D-arabinono-1,4-lactone oxidase (ALO)	Yeast and fungi: *C. albicans, S. cerevisiae*	D-arabinono-1,4-lactone to erythroascorbate
3. Aldono-lactonase	Mammals: mouse, human, rat	L-gulonic acid to L-gulono-1,4-lactone
4. L-gulonolactone oxidase (GLO)	Mammals: pig, mouse, rat. Also chicken.	L-gulono-1,4-lactone to ascorbate
5. GDP-mannose pyrophosphorylase	Plants: *A. thaliana*, Tobacco	D-mannose-1-Phosphate to GDP-D-mannose
6. GDP-mannose-3',5'-epimerase	Plants: *A. thaliana*	GDP-D-mannose to GDP-L-gulose OR GDP-L-galactose
7. L-galactose-1-phosphate phosphatase	Plants: *A. thaliana*, kiwifruit	L-galactose-1-phosphate to L-galactose
8. L-galactose dehydrogenase	Plants: *A. thaliana*, pea	L-galactose to L-galactono-1,4-lactone
9. L-galactono-1,4-lactone dehydrogenase (GALDH)	Plants: Cauliflower, sweet potato, tobacco	L-galactono-1,4-lactone to ascorbate
10. Inositol oxygenase	Plants: *A. thaliana*	*myo*-inositol to D-glucuronic acid
11. D-galacturonic reductase	Plants: *A. thaliana*, strawberry	D-galacturonic to L-galactonic acid

Table 6.1: Enzymes involved in ascorbate biosynthesis. Enzymes identified from fungi are coloured in blue, from mammals are coloured in red, and from plants are coloured in green.

6.1.3 Uptake of ascorbate

Cellular uptake of ascorbate is clearly of importance in humans and other species which cannot synthesise ascorbate *de novo*. However, a wide variety of organisms, including plants and mammals able to produce their own ascorbate, can also obtain ascorbate from their surroundings. Specific transporters able to mediate uptake in plants are yet to be identified (Horemans *et al.*, 2000). In addition to the mammalian ascorbate transporters described below, a bacterial operon that encodes several proteins that regulate ascorbate uptake and metabolism in *E. coli* has recently been identified (Yew and Gerlt, 2002; Zhang *et al.*, 2003). Although sgaT (UlaA), sgaA (UlaC) and sgaB (UlaB) are all required for ascorbate uptake (Zhang *et al.*, 2003), sgaT is thought be the actual transporter, which is predicted to include 12 transmembrane regions. In mammalian cells, two known routes of transport have been characterised: facilitated diffusion of dehydroascorbate primarily via glucose transporters (followed by reduction to ascorbate upon entry to the cell), and active transport of ascorbate itself, mediated by sodium-dependant transporters. These two mechanisms have been reviewed in detail elsewhere (Wilson, 2005).

Three separate glucose transporters have been shown to mediate dehydroascorbate uptake: GLUT1 and GLUT3 (Rumsey *et al.*, 1997) (from rat and human respectively, characterised in hamster and xenopus cells) and rat GLUT4 (characterised in rat and xenopus cells) (Rumsey *et al.*, 2000). Dehydroascorbate was completely reduced to ascorbate following uptake by GLUT1 and GLUT3 (Rumsey *et al.*, 1997). Intracellular ascorbate levels increased when either of these transporters was over-expressed and transport was inhibited by glucose. However, it should be noted that glucose does not completely inhibit dehydroascorbate uptake in all cell types (Himmelreich *et al.*, 1998; Daskalopoulos *et al.*, 2002), leading to speculation that other, as yet unidentified, transporters can also transport oxidised ascorbate (Wilson, 2005).

The second route of ascorbate uptake by mammalian cells is mediated by the sodium-dependant transporters SVCT1 and SVCT2, which exhibit high affinity transport of ascorbate (Daruwala *et al.*, 1999; Tsukaguchi *et al.*, 1999). The amino acid sequences of the two transporters, as well as the activities, are very similar, although the mammalian expression profiles differ. Both transporters are highly specific for ascorbate and were found not to significantly mediate the uptake of several compounds including dehydroascorbate and L-gulono-1,4-lactone (Tsukaguchi *et al.*, 1999). When SVCT2 expression was decreased by antisense RNA expression, ascorbate uptake decreased (Seno *et al.*, 2004). These sodium-dependant channels are thought to be more important than the GLUT channels for ascorbate uptake *in vivo*, as extracellular ascorbate is primarily in the reduced from, and because uptake by GLUT transporters is inhibited by glucose (Tsukaguchi *et al.*, 1999; Liang *et al.*, 2001).

6.1.4 Ascorbate recycling

As discussed, ascorbate molecules participate in a wide variety of cellular reactions, many of which involve counteracting oxidative stress. When ascorbate is oxidised – for example as a result of ascorbate peroxidase or oxidase activity – monodehydroascorbate (MDHA) is formed. MDHA is an unstable free radical which can be enzymatically reduced by MDHA reductase (MDHAR) to form ascorbate, or disproportionates to ascorbate and dehydroascorbate (DHA). In turn DHA can be reduced by DHA reductase (DHAR) enzymatically to ascorbate. These mechanisms, described in more detail below, have been previously summarised (Arrigoni and De Tullio, 2002) and are represented diagrammatically in figure 6.2. The reactions are often referred to as the ascorbate-glutathione cycle as DHARs use GSH. If DHA is not reduced it is degraded, although the exact degradation pathway and products are not yet clear.

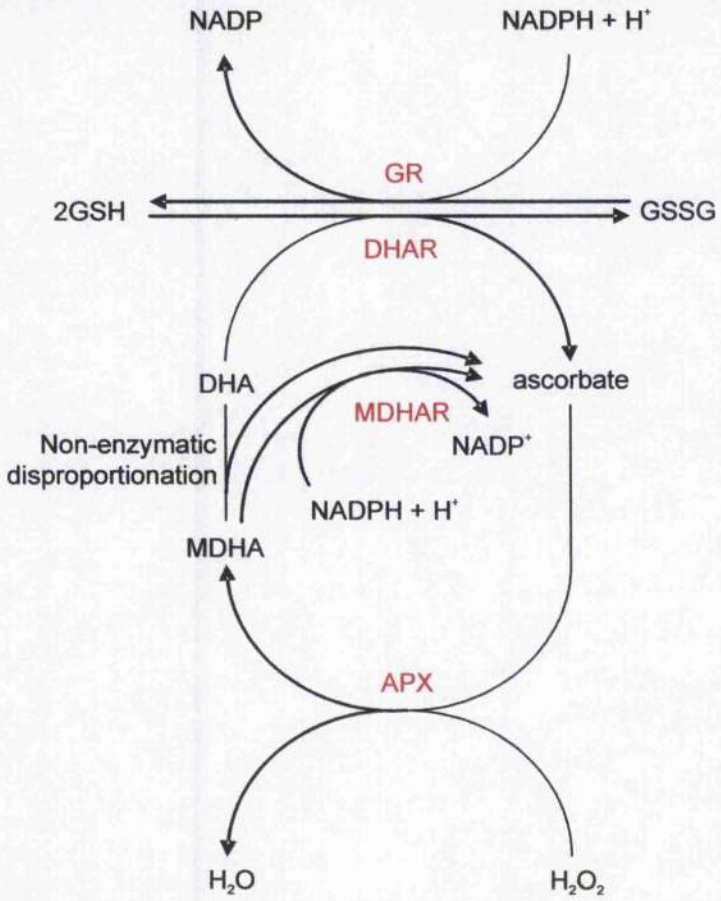

Figure 6.2: Ascorbate recycling via the ascorbate-glutathione cycle. Enzymes are shown in red: GR, glutathione reductase; DHAR, dehydroascorbate reductase; MDHAR, monodehydroascorbate reductase; APX, ascorbate peroxidase. Not all reactions are depicted stoichiometrically. Figure redrawn from Noctor and Foyer 1998.

MDHAR is a relatively uncharacterised enzyme despite its important role in maintaining reduced ascorbate in the cell. NADH/NADPH-dependant MDHAR activity was first detected in plant chloroplast extracts (Marre and Arrigoni 1958) and has since been found in a wide range of organisms. The enzyme responsible for the activity has been purified from several species including soybean (Dalton *et al.,* 1992) and cucumber (Hossain and Asada 1985) and is a FAD-binding protein. More recently the gene encoding MDHAR has been cloned (Murthy and Zilinskas 1994, Sano *et al.,* 2005) and analysed: the amino acid sequences, especially those of plant MDHARs, share similarity with some prokaryotic flavoenzymes (Murthy and Zilinskas 1994). Both N- and C- terminal targeting regions have been found in the various sequences which explain the diverse range of sub-cellular localisation attributed to the protein (Lisenbee *et al.,* 2005; Sano *et al.,* 2005). Treatment with various different sources of oxidative stress (including paraquat) was shown to up-regulate the expression of cabbage MDHAR protein (Yoon *et al.,* 2004), suggesting that the protein may be important for maintaining optimal ascorbate levels.

DHAR activity has been attributed to several different categories of proteins, including glutaredoxins and omega GSTs, as described in chapter one. In addition to these, the activity has been reported in protein disulphide isomerase (Wells *et al.*, 1990), thioredoxin (Trumper *et al.*, 1994), 3 alpha-hydroxysteroid dehydrogenase (Del Bello *et al.*, 1994), GSH-peroxidase (Washburn and Wells, 1999), and a trypsin-inhibitor (Trumper *et al.*, 1994). Interestingly, the CXXC motif found in dithiol glutaredoxins is present in the majority of these proteins; however, their amino acid sequences are otherwise diverse. Studies have shown that DHAR activity impacts intracellular ascorbate levels: when DHAR (an omega-GST like enzyme) was over-expressed in tobacco and maize, ascorbate levels rose accordingly (Chen *et al.*, 2003). A similar result was obtained in a study in which human omega GST was over-expressed in tobacco, and the plants also displayed increased tolerance to H_2O_2 and paraquat-induced oxidative stress (Kwon *et al.*, 2003).

6.1.5 Ascorbate in the trypanosomatids

The presence of both ascorbate and dehydroascorbate has been previously reported in *T. cruzi* epimastigotes. Furthermore, in both trypanosomes and *L. major*, ascorbate peroxidases have been previously identified and characterised (Wilkinson *et al.*, 2002a; Adak and Datta, 2005), the presence of which may be suggestive of ascorbate having an important role in the parasite. Indeed it was shown that the *T. cruzi* protein used ascorbate as an electron donor and reacted with H_2O_2 but not t-butyl hydroperoxide or cumene hydroperoxide *in vitro*. Whether the recombinant protein could also use the ascorbate analogue erythroascorbate, which is found in fungi, was not investigated. When the *T. cruzi* ascorbate peroxidase was over-expressed in the parasite, resistance was conferred to exogenous H_2O_2 with the parasites displaying a two-fold increase in resistance. Despite their exposure to high levels of oxidative stress due to the oxidative burst during invasion, trypanosomatids do not possess catalase (Boveris *et al.*, 1980), an important enzyme for the detoxification of H_2O_2 in many organisms. Thus an important role in protection against exogenous oxidative stress has been postulated for peroxidases, including this ascorbate-dependant protein (Adak and Datta, 2005). However, the localisation of the *T. cruzi* ascorbate peroxidase - and possibly the similar *Leishmania* enzyme due to the presence of a targeting sequence and transmembrane domain in the protein - to the endoplasmic reticulum (ER), may also implicate the protein in protection against endogenous H_2O_2. In *T. cruzi*, a second peroxidase has been localised to the ER (Wilkinson *et al.*, 2002b) and it has been postulated that it could protect the cell against reactive oxidant species generated here.

As mentioned, the dehydroascorbate reductase activity of TDR1 was one reason for investigating ascorbate in *Leishmania*. Over-expressing or knocking out TDR1 may have a knock-on affect on cellular ascorbate levels therefore ascorbate-dependant processes. Moreover if TDR1 was necessary for ascorbate recycling in *Leishmania* then loss of TDR1 could result in ascorbate biosynthesis being up-regulated to compensate for the lack of recycled material. However, it has previously been reported that the recycling of ascorbate from dehydroascorbate in *T. cruzi* is not mediated enzymatically and instead occurs spontaneously upon interaction of the oxidised compound with trypanothione (Krauth-Siegel and Ludemann, 1996). The authors were unable to detect any increase in ascorbate formation *in vitro* upon addition of crude parasite extract to the reaction mix which contained glutathione and dehydroascorbate. Regardless of whether TDR1 has a role in ascorbate recycling, the relative lack of published research on this interesting molecule in parasites was intriguing. Investigation into whether the parasites contained the machinery for, and were capable of, synthesising ascorbate would be the first step in elucidating the role of ascorbate in *Leishmania*, and is detailed in this chapter.

A very recently published report details an investigation into ascorbate synthesis in *T. brucei* (Wilkinson *et al.*, 2005). A sequence similar to L-gulonolactone oxidase, L-galactono-1,4-lactone dehydrogenase and D-arabinono-1,4-lactone oxidase, the final enzymes in the ascorbate biosynthetic pathways of mammals, plants and fungi respectively, was found in the parasite and designated TbALO. Recombinant TbALO was produced which was able to synthesise ascorbate from both L-galactono-1,4-lactone and D-arabinono-1,4-lactone, the plant and fungi substrates respectively. *T. brucei* bloodstream form parasites engineered to lack TbALO were viable and able to differentiate and infect mice, although they displayed a slight growth defect when cultured which was enhanced when ascorbate was depleted from the serum.

6.1.6 Aims of this study

In chapters three, four and five the discovery and characterisation of *L. major* TDR1 was described. Like oGST and glutaredoxins, with which TDR1 shares sequence similarity and active site motifs respectively, recombinant TDR1 displayed dehydroascorbate reductase activity *in vitro*. *L. major* TDR1 knockout parasites were hyper-sensitive to paraquat; a possible reason for this could be depleted ascorbate levels due to inefficient recycling of the oxidised form, caused by the absence of TDR1. As described, the maintenance of reduced ascorbate in trypanosomatids has been previously attributed to non-enzymatic

reduction by trypanothione; however, the possibility that TDR1 was capable of ascorbate recycling *in vivo* was intriguing.

Unlike many organisms, humans cannot synthesise ascorbate and instead scavenge it from their diet. Whether *Leishmania* also scavenge ascorbate, or whether they contain one of the several ascorbate biosynthetic pathways known to function in other life forms, was unknown. In order to clarify the situation we aimed to determine whether proteins similar to known ascorbate synthesis enzymes were present in *L. major*. In all ascorbate production pathways reported, a similar enzyme catalyses the final step: GLO in mammals, ALO in yeast and GALDH in plants. Therefore this protein in particular was of interest and it was our aim to ascertain whether *Leishmania* possessed such a protein and if so, to help determine its role in the parasite by altering its expression levels by genetic manipulation and analysing any resultant phenotype of the resultant mutants.

6.2 Results

6.2.1 Analysis of ascorbate biosynthesis homologues in *L. major*

As discussed in section 6.1.2 and illustrated in figure 6.1, several ascorbate and erythroascorbate biosynthetic pathways have been identified and various enzymes able to mediate reactions in the synthesis of ascorbate have been characterised; these are summarised in table 6.1. In order to determine whether *Leishmania* may possess such pathways, the amino acid sequences of these enzymes were used as enquiry sequences to search the *L. major* predicted protein database for putative ascorbate biosynthesis enzymes. As in chapter three, searches for *L. major* sequences were conducted using the omniblast feature at www.genedb.org and identified proteins were used as enquiry sequences to search known proteins of the original organism using the blastp facility found at http://www.ncbi.nlm.nih.gov/BLAST/. For each search the most significantly similar predicted protein to that of the enquiry sequence is described; less similar sequences are not described unless they displayed particularly high similarity. Alignments were performed using the alignX feature of vector NTI. The results of these blast searches and the ensuing analysis of the sequences identified are presented in this section and summarised in tables 6.2, 6.3 and 6.4.

6.2.1.1 GDP-mannose pyrophosphorylase

In the plant L-galactose pathway, the first enzyme known to be involved in ascorbate synthesis is GDP-mannose pyrophosphorylase which is responsible for the production of

GDP-D-mannose. As well as being involved in ascorbate synthesis (Conklin *et al.*, 1999; Keller *et al.*, 1999; Tabata *et al.*, 2002), the protein has an important role in the glycosylation of proteins and lipids and polysaccharide synthesis as GDP-D-mannose is required for these reactions. The *L. major* predicted protein database was searched for similar proteins using the 361 amino acid *A. thaliana* GDP-mannose pyrophosphorylase as an enquiry sequence. The most similar protein (p/n value = $1.2e^{-94}$) was the 379 amino acid LmjF23.0110, a previously characterised GDP-mannose pyrophosphorylase (Garami and Ilg 2001; Davis *et al.*, 2004). The protein sequence shares 50% identity and 70% conservation with that of the *A. thaliana* GDP-mannose pyrophosphorylase which was also the most alike *A. thaliana* protein to LmjF23.0110. Despite clear evidence that LmjF23.0110 is a GDP-mannose pyrophosphorylase, this is not implicit with it being involved in ascorbate synthesis due to its other known functions and published reports of LmjF23.0110 do not link it to ascorbate synthesis.

6.2.1.2 GDP-mannose-3',5'-epimerase

The plant enzyme which catalyses the subsequent reaction in ascorbate biosynthesis in this pathway – the conversion of GDP-D-mannose to GDP-L-galactose (or GDP-L-gulose) – is GDP-mannose-3',5'-epimerase. Again the activity of this protein may not be restricted to catalysing ascorbate synthesis (Wolucka *et al.*, 2001): GDP-L-galactose is a component of N-glycans and xyloglucans in plants and polysaccharides in invertebrates. The *A. thaliana* GDP-mannose-3',5'-epimerase 337 amino acid sequence was used as the enquiry sequence in the blast search. The most similar *L. major* protein (p/n value = $9.9e^{-8}$) was LmjF26.2230, an uncharacterised predicted protein 446 amino acids in length which is more than 100 amino acids longer than the *A. thaliana* protein. When the two sequences were aligned LmjF26.2230 had an N-terminal extension and several small N-terminal insertions as compared to the other protein. The protein sequence shares just 22% identity and 40% conservation with that of *A. thaliana* GDP-mannose-3',5'-epimerase. Moreover when LmjF26.2230 was used as an inquiry sequence for a blastp search of *A. thaliana* proteins, 41 sequences were more similar than the GDP-mannose-3',5'-epimerase with the most similar protein to LmjF26.2230 being annotated as a NADPH-dependant epimerase. Considering the low identity and conservation scores together with the results of the blastp search, it seems likely that LmjF26.2230 encodes a protein other than the GDP-mannose-3',5'-epimerase involved in ascorbate synthesis.

6.2.1.3 L-galactose-1-phosphate phosphatase

In this same plant pathway, the enzyme that converts GDP-L-galactose to L-galactose-1-phosphate has not yet been elucidated. However, the recently identified L-galactose-1-phosphate phosphatase catalyses the subsequent step: the dephosphorylation of L-galactose-1-phosphate to L-galactose (Laing *et al.*, 2004). As the authors allude to, this step is thought to be specific to ascorbate synthesis as L-galactose is not known to participate in any other cellular processes. The 279 amino acid *A. thaliana* sequence was used to search the *L. major* predicted protein database. Two sequences were highly similar to the enquiry protein: the 288 amino acid LmjF17.1390 (p/n value = $1.8e^{-38}$) and the 466 amino acid LmjF15.0880 (p/n value = $2.1e^{-26}$). LmjF17.1390 shares 35% identity and 55% conservation with *A. thaliana* L-galactose-1-phosphate phosphatase while LmjF15.0880 shares 32% identity and 49% conservation. Alignments between the amino acid sequences and that of the *A. thaliana* protein showed that while LmjF17.1390 was similar to the plant protein, LmjF15.0880 displayed both N- and C-terminal extensions as well as several large insertions, explaining the differences in amino acid lengths. Like the *A. thaliana* (and kiwifruit) enzyme, the *L. major* sequences are both annotated as being putative *myo*-inositol-1-phosphate phosphatases although the plant enzymes have now been shown to have much higher activity to L-galactose-1-phosphate than to *myo*-inositol-1-phosphate (Laing *et al.*, 2004). Given the high similarity between LmjF17.1390 and the *A. thaliana* protein, together with the fact that the *L. major* protein is more similar to it than any other *A. thaliana* protein, it is likely that LmjF17.1390 protein is a L-galactose-1-phosphate phosphatase. Due to the differences in size between the plant protein and LmjF15.0880 and the large insertions that appear to be present in the *L. major* protein based on the alignment with the *A. thaliana* sequence, it is unclear whether LmjF15.0880 may also act as a L-galactose-1-phosphate phosphatase. Interestingly, a blast search of the mouse protein database revealed there to be a very similar protein to the *A. thaliana* L-galactose-1-phosphate phosphatase (identification number NP061352) and this will be discussed in section 6.3 of this chapter. An alignment of this mouse sequence, together with *A. thaliana* L-galactose-1-phosphate phosphatase and LmjF17.1390 is shown in figure 6.3.

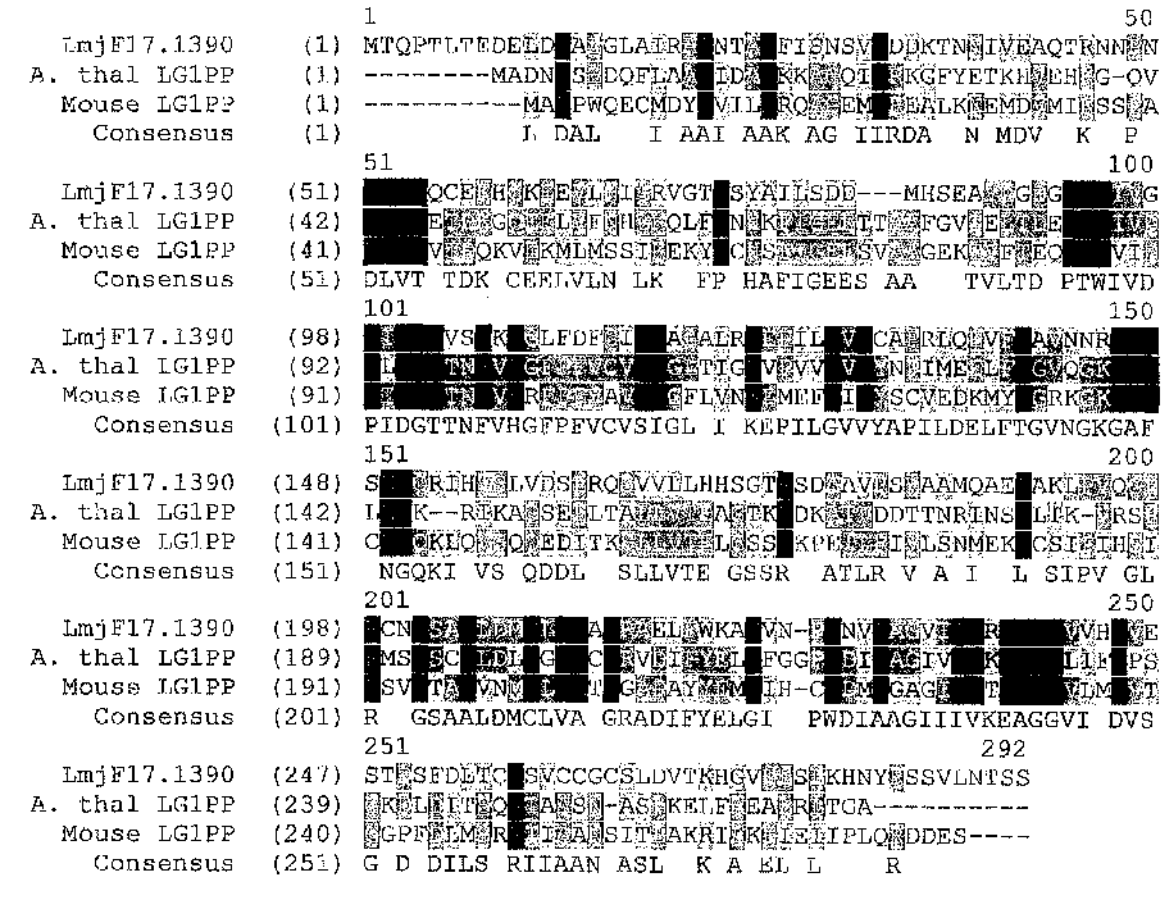

Figure 6.3: Alignment of *A. thaliana* L-galactose-1-phosphate phosphatase with the most similar *L. major* sequence, LmjF17.1390, and a putative mouse L-galactose-1-phosphate phosphatase. A. thal LG1PP and mouse LG1PP – *A. thaliana* and putative *Mus musculus* L-galactose-1-phosphate phosphatase (identification number NP061352), respectively. Dashes indicate gaps in the alignment. Identical residues are printed in white on a black background, conserved residues are printed in white on a grey background and similar residues are printed in black on a grey background.

6.2.1.4 L-galactose dehydrogenase and D-arabinose dehydrogenase

The plant enzyme that catalyses the next step in ascorbate synthesis via the L-galactose pathway is L-galactose dehydrogenase, which is similar in both sequence and function to the yeast erythroascorbate synthesis protein D-arabinose dehydrogenase. These enzymes catalyse the conversion of L-galactose to L-galactono-1,4-lactone and of D-arabinose to D-arabinono-1,4-lactone, respectively, although the yeast enzyme can use also L-galactose as a substrate. The amino acid sequences of *A. thaliana* L-galactose dehydrogenase and the *S. cerevisiae* D-arabinose dehydrogenase were used as enquiry sequences in blastp searches: the two most similar proteins to the plant sequence were the 286 amino acid LmjF31.2880 (p/n value = $1.5e^{-10}$) and the 279 amino acid LmjF32.0460 (p/n value = $3.1e^{-10}$) and the two most similar to the yeast sequence were LmjF32.0460 (p/n value = $1.2e^{-42}$) together with the 285 amino acid LmjF31.2150 (p/n value = $1.5e^{-42}$). LmjF31.2880 and LmjF32.0460

share 20% and 23% identity, respectively, and 40% and 41% conservation, respectively, with *A. thaliana* L-galactose dehydrogenase. Reflecting these relatively low scores, when the *L. major* sequences were used as enquiry sequences to search the *A. thaliana* protein database, there were 35 more similar proteins to LmjF32.0460 and 28 more similar proteins to LmjF31.2150 than the L-galactose dehydrogenase. Meanwhile LmjF32.0460 and LmjF31.2150 share 37% and 35% identity, respectively, and both share 54% conservation with *S. cerevisiae* D-arabinose dehydrogenase. However, again the single organism blast searches show that these predicted proteins are more similar to *S. cerevisiae* proteins other than D-arabinose dehydrogenase: 4 other proteins were more similar to LmjF32.0460 and 3 were more alike LmjF31.2150. LmjF32.0460 is similar to both the yeast and plant sequences; this was expected due to the similarity between the two proteins although the p/n scores together with the identity and conservation percentages clearly show that LmjF32.0460 is most similar to the yeast enzyme. LmjF31.2150 is also much more like D-arabinose dehydrogenase than LmjF31.2880 is to L-galactose dehydrogenase. However, the results of the single organism blast searches suggest that none of these four *L. major* sequences actually encode proteins involved in ascorbate biosynthesis as they are all more similar to proteins with different functions.

6.2.1.5 D-galacturonic acid reductase

Interestingly, a blast search using strawberry D-galacturonic acid reductase as the enquiry sequence identified the same *L. major* proteins as the searches using the *A. thaliana* L-galactose dehydrogenase and *S. cerevisiae* D-arabinose dehydrogenase sequences. D-galacturonic reductase operates in a separate plant ascorbate synthesis pathway catalysing the conversion of D-galacturonic to L-galactonic acid, which is then converted to L-galactono-1,4-lactone, and has not previously been shown to be similar to these other ascorbate synthesis proteins. The most similar proteins to the 319 amino acid strawberry D-galacturonic reductase were LmjF31.2150 (p/n value = $2.0e^{-39}$) and LmjF32.0460 (p/n value = $9.5e^{-33}$). LmjF31.2150 shares 37% identity and 56% conservation with strawberry D-galacturonic reductase while LmjF32.0460 shares 38% identity and 59% conservation. Alignments between the amino acid sequences and that of the strawberry protein showed that the *L. major* sequences both contained small deletions as compared to the other sequence, reflecting the 35/40 residue size difference. When the sequences were used to blast search the *A. thaliana* protein database there were two proteins more similar to the *L. major* sequences than the D-galacturonic reductase with the most alike protein in each case being annotated as a putative oxidoreductase. Therefore, despite the fact that LmjF31.2150 and LmjF32.0460 are both more similar to D-galacturonic reductase than to L-galactose

dehydrogenase or D-arabinose dehydrogenase, they may encode proteins that are not involved in ascorbate biosynthesis. This seems even more likely for LmF31.2150, which has been experimentally shown to possess prostaglandin f2-alpha synthase activity (Kabututu *et al.*, 2003).

6.2.1.6 Aldono-lactonase

The enzyme that catalyses the penultimate step in ascorbate synthesis in mouse – and presumably therefore in other mammals that synthesise ascorbate via the uronic acid pathway – was recently identified as an aldono-lactonase which is not similar to the proteins performing this step in other organisms described in the previous two paragraphs. Accordingly, the *L major* sequence most similar to the 299 amino acid mouse sequence was the 413 amino acid LmjF28.1230 (p/n value = $1.4e^{-15}$), not a protein identified in a previous blast search. LmjF28.1230 shares 24% identity and 43% conservation with the amino acid sequence of the mouse aldono-lactonase; an alignment of the two sequences is shown in figure 6.4. Alignment of the two proteins showed that the *L major* sequence had several small insertions, together with a small C-terminal extension in comparison to the mouse sequence. However when LmjF28.1230 was used as an inquiry sequence in a blastp search of mouse proteins, the most similar was the aldono-lactonase. This is intriguing, as despite the relatively low identity and conservation scores, and the discrepancies between the alignments of the two sequences, LmjF28.1230 is not more similar to any other mouse protein. It is also annotated as a sequence orphan in the *L. major* database. Therefore it remains possible that LmjF28.1230 does encode an aldono-lactonase involved in ascorbate biosynthesis.

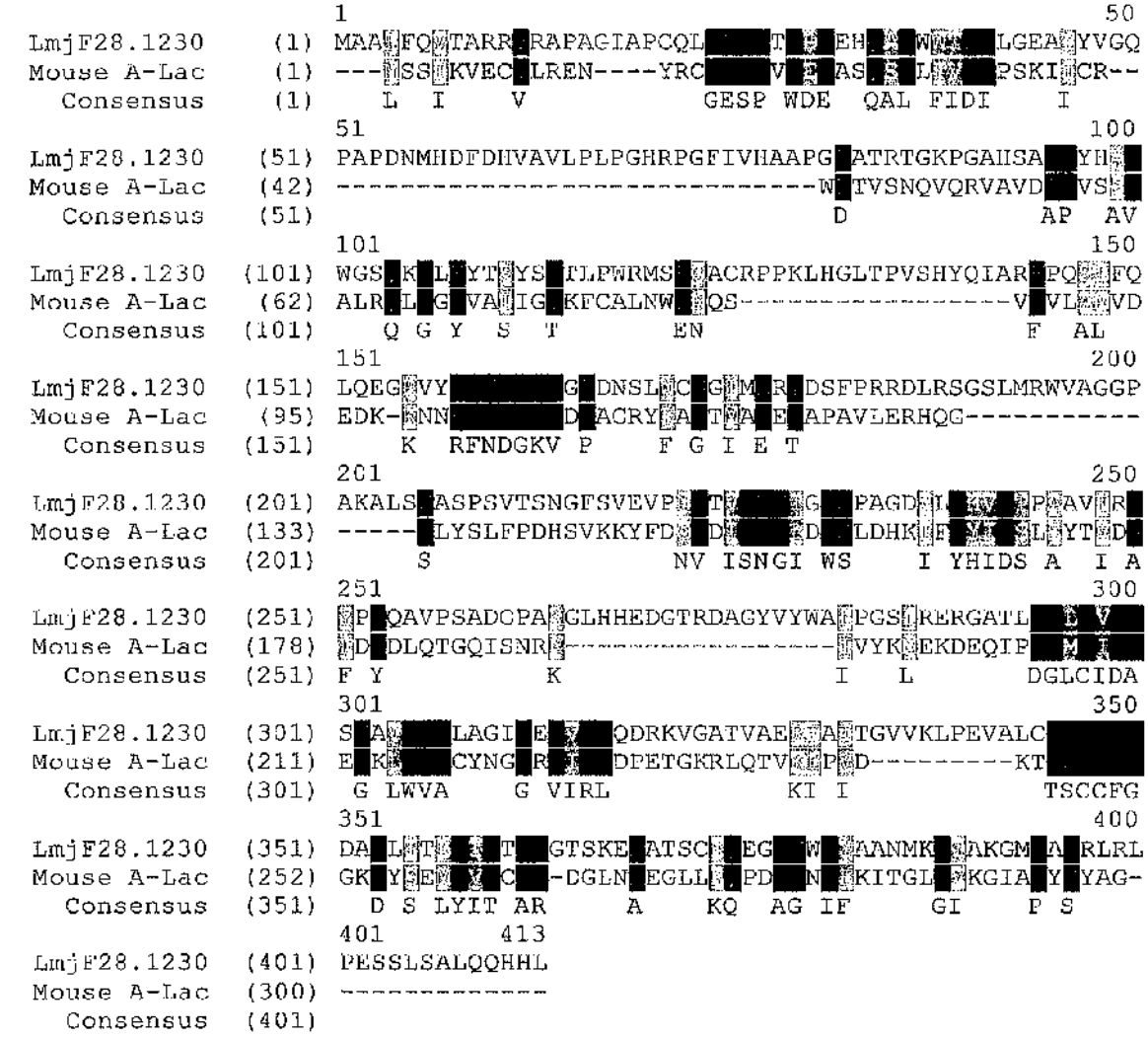

Figure 6.4: Alignment of mouse aldono lactonase with the most similar *L. major* sequence, LmjF28.1230. Mouse A-lac – *Mus musculus* aldono lactonase. Dashes indicate gaps in the alignment. Identical residues are printed in white on a black background and similar residues are printed in black on a grey background.

6.2.1.7 GLO, ALO and GALDH

The final step in ascorbate production in the mammalian, yeast and the plant L-galactose and D-galacturonic acid pathways is catalysed by a similar enzyme: GLO in mammals, ALO in yeast and GALDH in plants. Representative sequences of all three proteins – the 440 amino acid pig GLO, the 526 amino acid *S. cerevisiae* ALO and the 610 amino acid *A. thaliana* GALDH - were used in blast searches of *L. major* predicted proteins and the same sequence was identified in each: LmjF17.1360 (p/n values = $9.6e^{-53}$, $3.2e^{-25}$ and $6.7e^{-22}$, respectively). LmjF17.1360, which is 502 amino acids in length, shares 28% identity and 54% conservation with pig GLO, 19% identity and 38% conservation with *S. cerevisiae* ALO, and 20% identity and 42% conservation with *A. thaliana* GALDH. Alignments between the sequences, shown in figure 6.5, reveal that LmjF17.1360 has a small C-

terminal extension and a 55 amino acid insertion in comparison to the pig GLO, is lacking a 100 amino acid N-terminal extension when compared to the *A. thaliana* GALDH and aligns poorly with *S. cerevisiae* ALO in the middle of the sequences. When LmjF17.1360 was used as an enquiry sequence to search the protein databases of pig, *A. thaliana* and *S. cerevisiae*, the *L. major* sequence was most like the GLO, GALDH and ALO respectively. This suggests that LmjF17.1360 is likely to encode a protein capable of mediating the final step in ascorbate biosynthesis in *Leishmania*. Given the much higher p/n value and identity and conservation scores between it and the pig sequence rather than those of plant or yeast, it appears that LmjF17.1360 is most closely related to GLO and will be herein referred to as LmGLO. This sequence shares 45% identity and 64% conservation with the recently identified *T. brucei* enzyme TbALO (Wilkinson *et al.*, 2005) described in section 6.1.5 of this chapter. However, based on substrate preference, the authors concluded that the enzyme is most similar to ALO or GALDH rather than GLO, which contrasts with which enzyme it is most closely related to based on amino acid sequence similarity alone.

6.2.1.8 Inositol oxygenase

Inositol oxygenase is the only other protein involved in ascorbate synthesis to have been identified and characterised to date. The protein is thought to participate in an alternative pathway in plants, converting *myo*-inositol to D-glucuronic acid. The *L. major* predicted protein database was searched with the 317 amino acid sequence of *A. thaliana* inositol oxygenase and the most similar protein (p/n value 0.51) was the 378 amino acid LmjF18.1380. LmjF18.1380 shares just 14% identity and 32% conservation with the *A. thaliana* protein, poorly aligns with the sequence and has N- and C- terminal extensions compared to it. When the *L. major* sequence was used to search the *A. thaliana* protein database there were 38 more similar proteins to it than the inositol oxygenase; the most alike was a pyruvate dehydrogenase which was also what LmjF18.1380 was annotated as in the *L. major* database. Taking all this information together, it is highly unlikely that LmjF18.1380 encodes an inositol oxygenase involved in ascorbate synthesis.

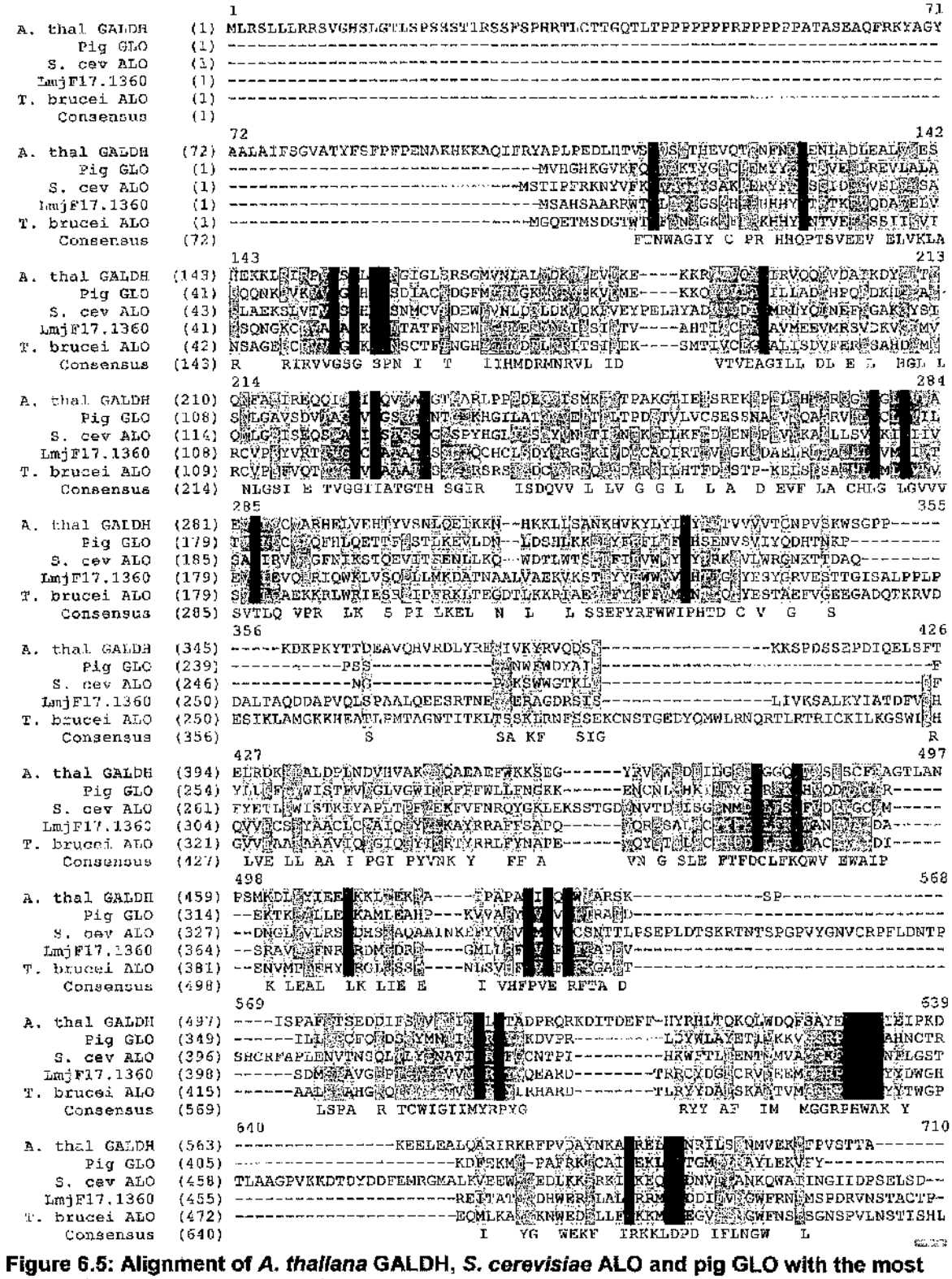

Figure 6.5: Alignment of *A. thaliana* GALDH, *S. cerevisiae* ALO and pig GLO with the most similar *L. major* sequence, LmjF17.1360, and the similar trypanosome enzyme, *T. brucei* ALO. A. thal GALDH – *A. thaliana* GALDH, S. cev ALO – *S. cerevisiae* ALO, pig GLO – *Sus scrofa* GLO. The putative *T. brucei* ALO C-terminal SHL signalling tag which is absent in the *L. major* sequence is underlined in red. Dashes indicate gaps in the alignment. Identical residues are printed in white on a black background, conserved residues are printed in white on a grey background and similar residues are printed in black on a grey background.

This analysis does not provide any definitive proof of which ascorbate pathways, if any, are present in *L. major*. However, it seems reasonable to conclude that some of the sequences identified may have a role. LmjF17.1390 is very like the *A. thaliana* L-galactose-1-phosphate phosphatase. Meanwhile LmjF28.1320 and LmjF17.1360 are similar to the only known enzymes that mediate the mammalian ascorbate biosynthesis pathway: the recently identified mouse aldono-lactonase and pig GLO. Crucially, these *L. major* sequences were more similar to ascorbate biosynthesis enzymes, which have no other known function, than any other proteins in the species the original enquiry sequences were derived from. Thus, they were judged to be the most likely to be involved in ascorbate synthesis in *Leishmania*. The importance of GLO in ascorbate synthesis in mammals is paramount: humans lack this enzyme and therefore have to derive ascorbate from their diet. Whether LmGLO has a similar role in the parasite was investigated, and is discussed herein.

Enquiry sequence: organism, enzyme name, and amino acid length	***Leishmania* homologues, p/n scores and number of amino acids**	**% identity, % conservation and observations on alignments**	**Single organism back BLAST results**
1. *S. cerevisiae* D-arabinonose dehydrogenase (NP009707), 344 amino acids	**a. LmjF32.0460** $1.2e^{-42}$, 279 amino acids **b. LmjF31.2150** $1.5e^{-42}$, 285 amino acids	**a.** 37%, 53%. N-term more alike. **b.** 35%, 54%. L. maj seq 30 amino acid gap 137-167.	**a.** 5th hit to enq seq. Best is glycerol dehydrogenase. **b.** 4th hit to enq seq. Best is as above.
2. *S. cerevisiae* D-arabinono-1,4-lactone oxidase (NP013624), 526 amino acids	**LmjF17.1360** $3.2e^{-25}$, 502 amino acids	19%, 38%. Middle of sequences display poor similarity.	Best hit to enq seq.

Table 6.2: *L. major* sequences similar to ascorbate biosynthesis enzymes in fungi. Abbreviations used are L. maj – *L. major*, seq – sequence, ext – extension, N-term – N-terminal, C-term – C terminal, enq – enquiry. Numbers in first column correlate with those in figure 6.1.

Enquiry sequence: organism, enzyme name, and amino acid length	***Leishmania* homologues, p/n scores and number of amino acids**	**% identity, % conservation and observations on alignments**	**Single organism back BLAST results**
3. Mouse aldono lactonase (NP033086), 299 amino acids	**LmjF28.1230** $1.4e^{-15}$, 413 amino acids	24%, 43%. Six small gaps in mouse seq. L. maj seq also has small C-term ext.	Best hit to enq seq.
4. Pig L-gulonolactone oxidase (Q8HXW0), 440 amino acids	**LmjF17.1360** $9.6e^{-53}$, 502 amino acids	28%, 54%. Pig seq. contains a 55 amino acid gap. 235-290. L. maj seq has small C-term ext.	Best hit to enq seq.

Table 6.3: *L. major* sequences similar to ascorbate biosynthesis enzymes in animals. Abbreviations used are L. maj – *L. major*, seq – sequence, ext – extension, N-term – N-terminal, C-term – C terminal, enq – enquiry. Numbers in first column correlate with those in figure 6.1.

Enquiry sequence: organism, enzyme name, and amino acid length	***Leishmania* homologues, p/n scores and number of amino acids**	**% identity, % conservation and observations on alignments**	**Single organism back BLAST results**
5. *A. thaliana* GDP-6-mannose pyrophosphorylase (AAC78474), 361 amino acids	**LmjF23.0110** $1.2e^{-94}$, 379 amino acids	50%, 70%. Region between 250-270 displays poor similarity.	Best hit to enq seq.
6. *A. thaliana* GDP-mannose-3',5'-epimerase (CAD7OO55), 377 amino acids	**Lmj26.2230** $9.9e^{-8}$, 446 amino acids	22%, 40%. L. maj seq has N-term ext. Also, gaps in A. thaliana N-term	42^{nd} hit to enq seq. Best is NADPH-dependant epimerase/dehydrogenase.
7. *A. thaliana* L-galactose-1-phosphate phosphatase (NP001030626), 269 amino acids	**a. LmjF17.1390** $1.8e^{-38}$, 288 amino acids **b. LmjF15.0880** $2.1e^{-26}$, 466 amino acids	**a.** 35%, 55%. Good similarity. **b.** 32%, 49%. Large gaps in A. thaliana seq. N- and C- term ext. in L. maj seq.	**a.** Best hit to enq seq. **b.** Best hit to enq seq.
8. *A. thaliana* L-galactose dehydrogenase (CAD10386), 319 amino acids	**a. LmjF31.2880** $1.5e^{-10}$, 286 amino acids **b. LmjF32.0460** $3.1e^{-10}$, 279 amino acids	**a.** 20%, 40%. Better similarity in N-term. **b.** 23%, 41%. Quite poor overall.	**a.** 36^{th} hit to enq seq. Best is oxidoreductase. **b.** 29^{th} hit to enq seq. Best is as above.
9. *A. thaliana* L-galactono-1,4-lactone dehydrogenase (NP190376), 610 amino acids	**LmjF17.1360** $6.7e^{-22}$, 502 amino acids	20%, 42%. L. maj seq lacks first 100 amino acids.	Best hit to enq seq.
10. *A. thaliana* inositol oxygenase (AAP59548), 317 amino acids	**LmjF18.1380** 0.51, 378 amino acids	14%, 32%. L. maj seq N- and C-term ext. Also small gaps in A. thaliana seq.	39^{th} hit to enq. seq. Best is pyruvate dehydrogenase.
11. Strawberry D-galacturonic reductase (AAT76306), 319 amino acids	**a. LmjF31.2150** $2.0e^{-39}$, 284 amino acids **b. LmjF32.0460** $9.5e^{-33}$, 279 amino acids	**a.** 37%, 56%. Small gaps in L. maj seq. **b.** 38%, 59%. Small gaps in L. maj seq.	**a.** 3^{rd} hit to enq seq. Best is oxidoreductase **b.** 3^{rd} hit to enq seq. Best is oxidoreductase.

Table 6.4: *L. major* sequences similar to ascorbate biosynthesis enzymes in plants. Abbreviations used are L. maj – *L. major*, seq – sequence, ext – extension, N-term – N-terminal, C-term – C terminal, enq – enquiry. Numbers in first column correlate with those in figure 6.1.

6.2.2 LmGLO expression in *Leishmania*

In order to clarify whether LmGLO expression could be detected, western blots were performed on various *Leishmania* species promastigote lysates, using rat anti-sera that had been raised to recombinant LmGLO (figure 6.6). As well as *L. major*, *L. infantum* and *L mexicana* lysates were also used as sequences with 94% and 81% identity to LmGLO are present in the *L. infantum* and *L. braziliensis* protein databases, respectively. Both the first and final bleed batches of antisera were used for western blotting (figure 6.6A and 6.6B, respectively) but unfortunately both antibodies detected several different proteins. The first bleed antiserum detected the 57 kD 6-His LmGLO recombinant protein and in the *L. major* promastigote lysate a slightly smaller band that may be LmGLO (untagged LmGLO is predicted to be 56 kD). Unfortunately this could not be further investigated as all of the antiserum obtained from the first bleed was used in this experiment and when re-used did not detect either the recombinant protein or any proteins from *Leishmania* lysates. In addition to the ~56 kD band the first bleed antibody detected several larger proteins, which were presumed to be due to non-specific cross-reactivity of the anti-sera. The final bleed antiserum detected many proteins, both in the recombinant protein fraction and in the parasite lysates. One of the proteins detected appeared to be of around the expected size of LmGLO, however, despite repeated attempts to optimise the conditions of the western blot when using the antiserum, it was impossible to distinguish the band and discern any useful information regarding endogenous LmGLO expression.

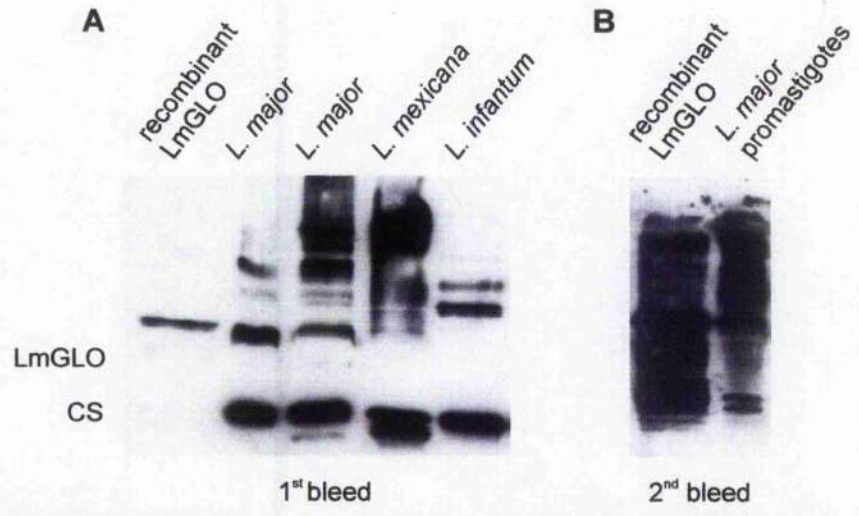

Figure 6.6: Western blot analysis of LmGLO expression in the soluble fraction of *Leishmania*. A – first bleed antiserum. A protein of a slightly smaller size than the annotated LmGLO recombinant protein is visible in the *L. major* lysates. B – second bleed antiserum. 10 µg-30 µg of parasite lysate was loaded per lane. CS, cysteine synthase, (control).

6.2.3 Creation of LmGLO over-expressing *L. major* promastigotes

The sequence encoding LmGLO was amplified from *L. major* genomic DNA, sub-cloned into the pGEM T-easy and then cloned into the pGL102 plasmid. The plasmid with the *LmGLO* gene was named pGL102LmGLO; maps of this and the empty vector are shown in figure 6.7. The pGL102 plasmid is a *Leishmania* episomal expression vector and contains the neomycin resistance gene as a selectable marker. Uncut pGL102 and pGL102LmGLO were transfected into *L. major* promastigotes which were subsequently incubated in culture with neomycin so that only successful transformed parasites would grow. The transformed parasite lines will be herein referred to by the names of the vectors they were transformed with.

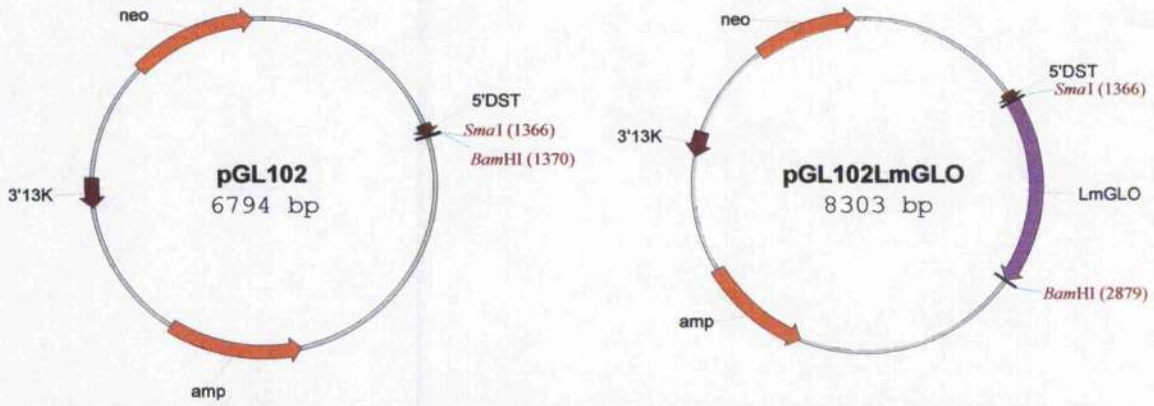

Figure 6.7: Plasmids for the over-expression of LmGLO in *L. major* promastigotes. Schematic representation of the pGL102 plasmid for the over-expression of LmGLO in *Leishmania*. A – empty pGL102 vector, for use as a negative control. B – pGL102 + LmGLO (pGL102LmGLO) for the over-expression of LmGLO. amp, ampicillin resistance gene; neo, neomycin resistance gene. The restriction sites used to clone LmGLO into the vector are shown in dark red.

6.2.4 Phenotype analysis of *L. major* putative LmGLO over-expressing lines

Due to the failure of the anti-LmGLO antiserum to detect LmGLO specifically, it was not possible to confirm the over-expression of LmGLO in the parasites and the phenotypic analysis presented in this section must be regarded in this context.

6.2.4.1 Morphology and growth of promastigotes

Parasites putatively over-expressing LmGLO were examined microscopically and no morphological differences could be seen between them and the parasites containing the empty pGL102 vector. After several passages with neomycin present in the culture media, the pGL102 and pGL102LmGLO lines were counted and diluted to the same concentration. Thereafter their growth was monitored by daily counting (figure 6.8). The parasite lines were found to grow at similar rates.

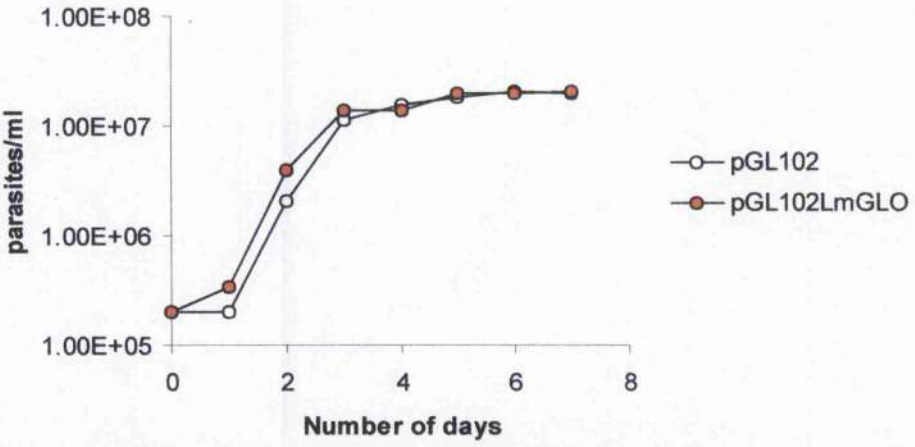

Figure 6.8: Growth curves of pGL102 and pGL102LmGLO *L. major* promastigotes. Cultures were seeded at a concentration of 2 x 10^5 parasites/ml and were counted daily. Both lines were grown with 50 µg/ml neomycin.

6.2.4.2 *In vitro* infectivity to macrophages

The ability of the different stationary phase promastigote parasite lines to infect peritoneal macrophages was assessed (figure 6.9). pGL102LmGLO infected a higher percentage of macrophages than either the pGL102 or WT lines: approximately 4 times and twice as many, respectively. This suggests that if pGL102LmGLO parasites are indeed over-expressing LmGLO then this aids infection. However, the differences observed were not found to be significant, likely to be due to the lack of repetitions of this experiment: further investigation is required. The variation between the infection rates between the WT and pGL102 lines was also of concern as they would be expected to infect at a similar rate. It is possible that the pGL102 line have been adversely affected by the transformation procedure, causing them to be less infective *in vitro*.

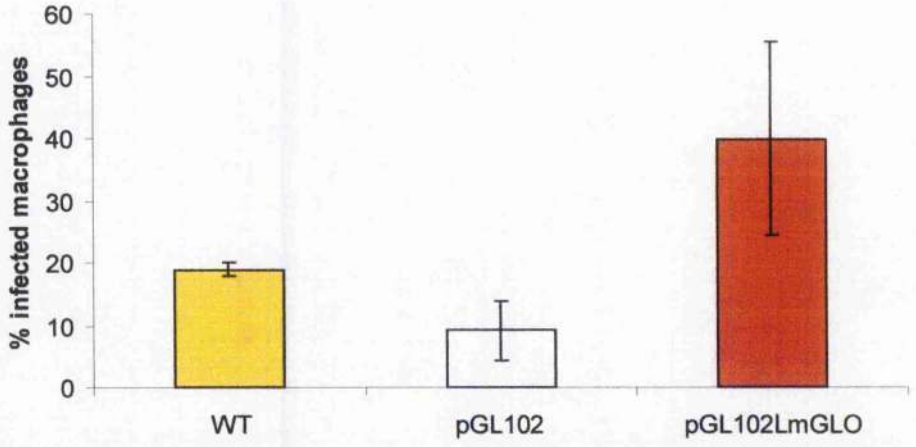

Figure 6.9: Infectivity of WT, pGL102 and pGL102LmGLO *L. major* promastigotes to macrophages. Stationary phase promastigotes were used to infect peritoneal macrophages at a rate of 2:1 and the slides were incubated for 5 days post-infection. Results are the means ± SE from two experiments. The WT and pGL102LmGLO lines did not infect the macrophages at significantly different rates ($p > 0.05$).

6.2.4.3 *In vivo* infectivity to mice

To assess *in vivo* infectivity, pGL102LmGLO, pGL102 and WT stationary growth-phase promastigotes were inoculated into mouse footpads, the thicknesses of which were then measured over time (figure 6.10). The results broadly reflected those of the *in vitro* infectivity experiments: pGL102LmGLO parasites were responsible for the most rapid footpad growths which were slightly, but not significantly, faster than those infected with the WT parasites, while footpads infected with pGL102 parasites took approximately 2 weeks longer to reach similar thicknesses. Over-expression of LmGLO is from an episomal expression vector, the presence of which is assured by keeping the parasites under neomycin pressure. This is not possible during *in vivo* work and the number of episomes, and therefore the level of LmGLO expression, is likely decrease over time. This may explain why the increased infectivity of pGL102LmGLO parasites as compared to the WT and pGL102 lines is not as marked as in the macrophage infectivity experiment, where infections are monitored over days as opposed to weeks. Once more the pGL102 parasites caused less virulent infections than the WT parasites, which again may be indicative of them being compromised in some way by the transformation procedure. This will be discussed in more detail in section 1.3.

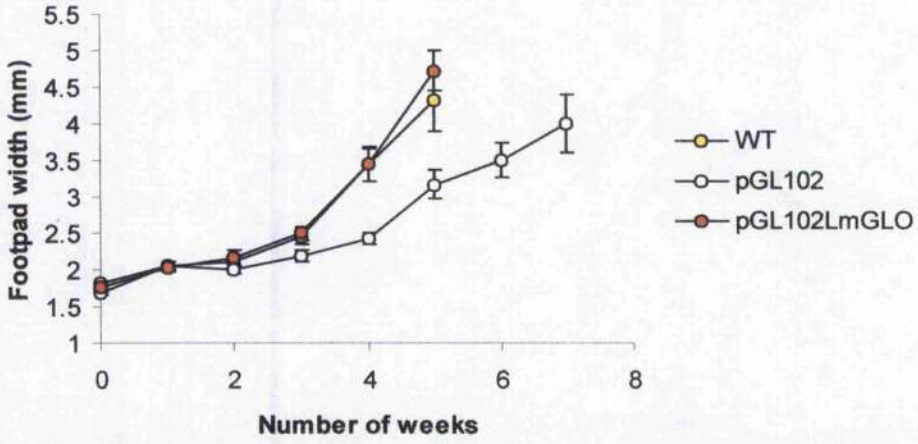

Figure 6.10: Infectivity of WT, pGL102 and pGL102LmGLO, *L. major* promastigotes to mice. 5×10^5 stationary-phase promastigotes were resuspended in 20 µl PBS and inoculated into one footpad of each BALB/C mouse. The footpad thicknesses were subsequently measured weekly, and the results are the means ± from five mice. The WT and pGL102LmGLO infections were not significantly different ($p > 0.05$).

6.2.5 Creation of *L. major LmGLO* knock-out parasite lines

The 3' and 5' flanking regions of LmGLO were amplified from *L. major* genomic DNA, sub-cloned into pET28a(+) and cloned into vectors pGL345 and pGL842 which contain hygromycin and blasticidin resistance genes, respectively (figure 6.11). The constructs containing the flanking regions and drug resistance markers were cut out and purified and the linear DNA was used to transform *L. major* promastigotes. Two independent lines, KOLmGLO3 and KOLmGLO8, were generated, both having undergone transformation with each of the constructs sequentially and grown up in the presence of the selection drugs. In addition, following the second transfection and overnight recovery period, the two independent cultures were divided and ascorbate was added to one of each to a final concentration of 10 mM. This was to ensure that it would be available if the loss of LmGLO resulted in the parasites having to exogenously source their ascorbate. KOLmGLO8 grew up to stationary phase regardless of whether ascorbate had been added to the media, while only the KOLmGLO3 culture that contained ascorbate grew up quickly. After several passages in the selective drugs, KOLmGLO3 and KOLmGLO8 clones were derived by setting up the cultures with ascorbate in 96-well plates at a concentration of approximately 0.1 parasites/well. Two clones were isolated from KOLmGLO3 – deemed KOLmGLOA1 and KOLmGLOA2 – and one clone was derived from KOLmGLO8: KOLmGLOB. All three clones took several weeks to grow up.

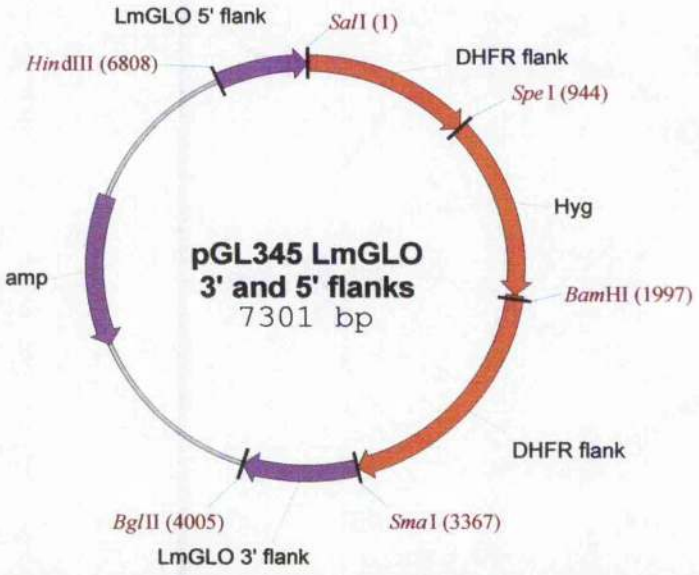

Figure 6.11: Construct for the gene knock-out of LmGLO in *L. major* promastigotes. Schematic representation of the pGL345 plasmid containing the LmGLO 3' and 5' flanking regions. The second plasmid used for the construction of the LmGLO knock-out was the pGL842 plasmid, which is exactly the same except that it contains a blasticidin rather than hygromycin resistance gene. Hyg, hygromycin resistance gene; amp, ampicillin resistance gene; DHFR flanks, flanking regions of the dihydrofolate reductase gene. The restriction sites used to clone the LmGLO flanks and hygromycin resistance gene into the vector are shown in dark red. *HindIII* and *BglII* were used to cut out the flank-containing linear construct for transfection into *L. major*.

Because it was not possible to ascertain whether LmGLO had been successfully knocked out by western blotting, a southern blot was performed on genomic DNA derived from KOLmGLOA1 and KOLmGLOA2 and KOLmGLOB, together with WT parasites. The DNA was digested with restriction enzymes that, depending on whether the WT or replacement allele was present, would yield fragments of different lengths containing the LmGLO 3' flanking region (figure 6.12A). The 3' flanking region was used as a probe and would hybridise to a fragment of approximately 1.4 kb if the WT allele was present, 2.4 kb if the blasticidin resistance gene was present and 3.7 kb if the hygromycin resistance gene was present. Unfortunately no fragments were detected in the WT and KOLmGLOA1 and KOLmGLOA2 digested DNA samples. However, fragments of approximately 2.4 kb and 3.7 kb were detected in the KOLmGLOB sample (figure 6.12B), corresponding to both resistance markers that would be present in a bona fide LmGLO knockout. Therefore it was concluded that KOLmGLOB lacks LmGLO. The absence of fragments detected in KOLmGLOA1 and KOLmGLOA2 suggest it is unlikely that they are genuine LmGLO knockouts although the experiment was inconclusive.

6.2.6 Phenotype analysis of *L. major LmGLO* knock-out parasite lines

The parent lines of the clones, KOLmGLO3 (which KOLmGLOA1 and KOLmGLOA2 were derived from) and KOLmGLO8 (which KOLmGLOB was derived from) were analysed in some experiments as the clones took a long time to obtain. As well as examining the phenotype of KOLmGLOB – deemed to be a genuine LmGLO knockout – KOLmGLOA1 and KOLmGLOA2 and their parent lines were also included in experiments. This was because, although unlikely to lack LmGLO, they would act as useful controls due to them having undergone the same transformation procedure and exposure to drugs as KOLmGLOB.

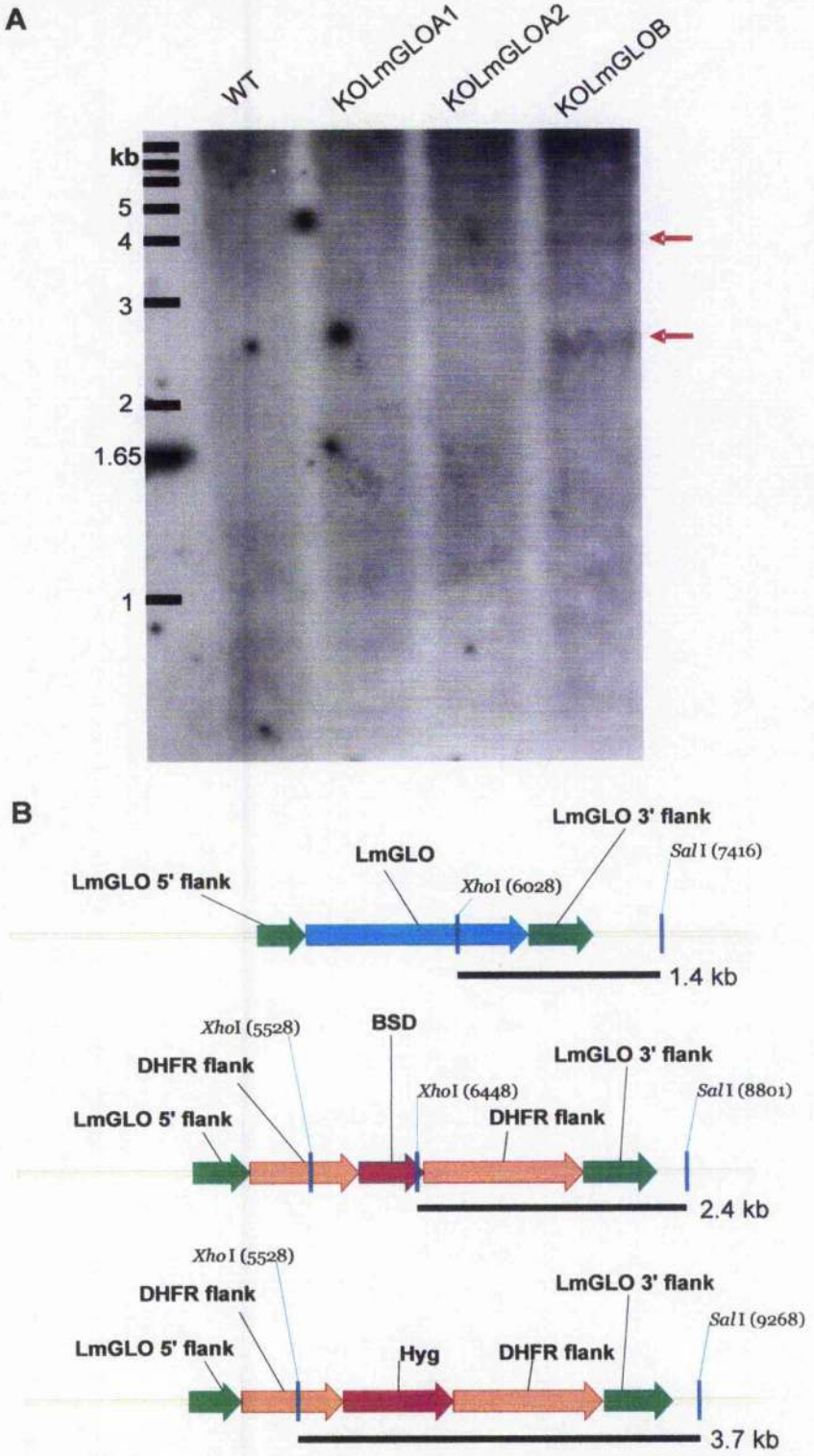

Figure 6.12: Southern blot analysis of the LmGLO locus of *L. major* KOLmGLO lines. A – Southern blot analysis of genomic DNA extracted from the different parasite lines as indicated above the gel image. The 2.4 kb and 3.7 kb bands visible in the lane containing DNA from the KOLmGLOB clone are indicated with red arrows. The blot was exposed to the film for 7 days. The positions and sizes of the molecular size markers are indicated to the left of the gel image; kb, kilo base pairs. B – schematic representation of the LmGLO locus on chromosome 17 (LmGLO shown in blue), and the altered locus following successful gene replacement with either the hyg (hygromycin) or bsd (blasticidin) resistance markers (shown in red). The expected sizes of the fragments following digestion of the genomic DNA with *XhoI* and *SalI*, to be detected by southern blot using the 3' flanking region as a probe, are indicated below each diagram and represented with a black line. The restriction sites and their relative positions are also shown.

6.2.6.1 Growth of promastigotes grown with and without exogenous ascorbate

As described, KOLmGLO3 and KOLmGLO8 were originally divided and grown with and without ascorbate (+asc and –asc) added to the culture media although only KOLmGLO3 with exogenous ascorbate thrived. After several passages, the growth rates of the remaining three cultures – KOLmGLO3+asc, KOLmGLO8-asc and KOLmGLO8+asc – were monitored as before (figure 6.13). The three parasite lines grew at similar rates although KOLmGLO3+asc grew slightly faster than KOLmGLO8+asc which grew faster than KOLmGLO8-asc. However the most marked difference between the cultures was in the concentrations the parasites reached when in the stationary phase of growth. After several days KOLmGLO3+asc grew to and remained at a density of ~2 x 10^7 parasites/ml, as WT parasites do under similar conditions. Meanwhile KOLmGLO8-asc and KOLmGLO8+asc reached concentrations of just ~0.7 x 10^6/ml and 0.9 x 10^6/ml respectively, even after 10 days in culture. KOLmGLOB, derived from KOLmGLO8+asc, was the only clone found to lack the LmGLO gene while the clones derived from KOLmGLO3+asc were shown to be unlikely to lack the gene. Therefore it is possible that the loss of LmGLO causes a growth defect in *L. major* promastigotes, causing the parasites to grow more slowly than usual and to fail to reach high densities. This is in accordance with the growth defect observed in bloodstream form *T. brucei* lacking the homologous gene to LmGLO (Wilkinson *et al.*, 2005). Also, the observation that KOLmGLO8-asc grows at a slightly slower rate and reaches lower parasite densities in culture than KOLmGLO8+asc suggests that the presence of exogenous ascorbate may have had a beneficial effect in the early stages of transformation.

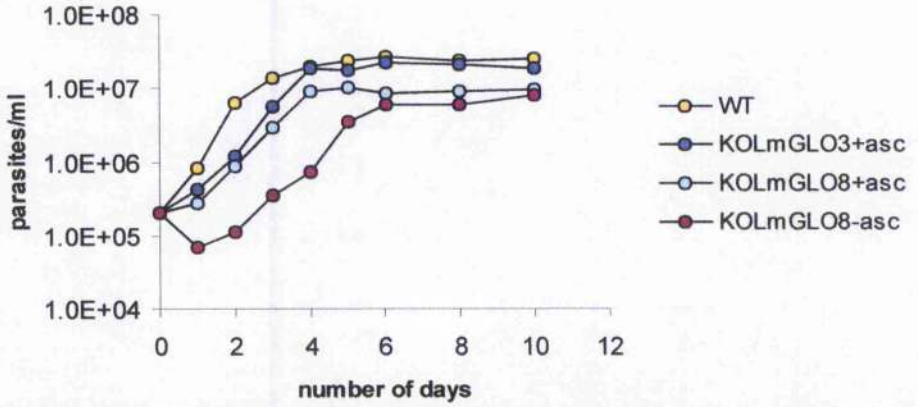

Figure 6.13: Growth curve of WT, KOLmGLO3+asc, KOLmGLO8+asc and KOLmGLO8-asc *L. major* promastigotes. Cultures were seeded at a concentration of 2 x 10^5 parasites/ml and were counted daily. All lines except WT parasites were grown with 15 µg/ml blasticidin and 100 µg/ml hygromycin. KOLmGLO3+asc and KOLmGLO8+asc were also grown with 1 mM ascorbate present.

The three cultures were also grown with and without added ascorbate to ascertain if its inclusion in the culture media affected the growth rates of the parasites after they had been in culture for several passages (figure 6.14). Exogenous ascorbate had no affect on the growth of any of the cultures at this stage and was subsequently omitted from the culture media.

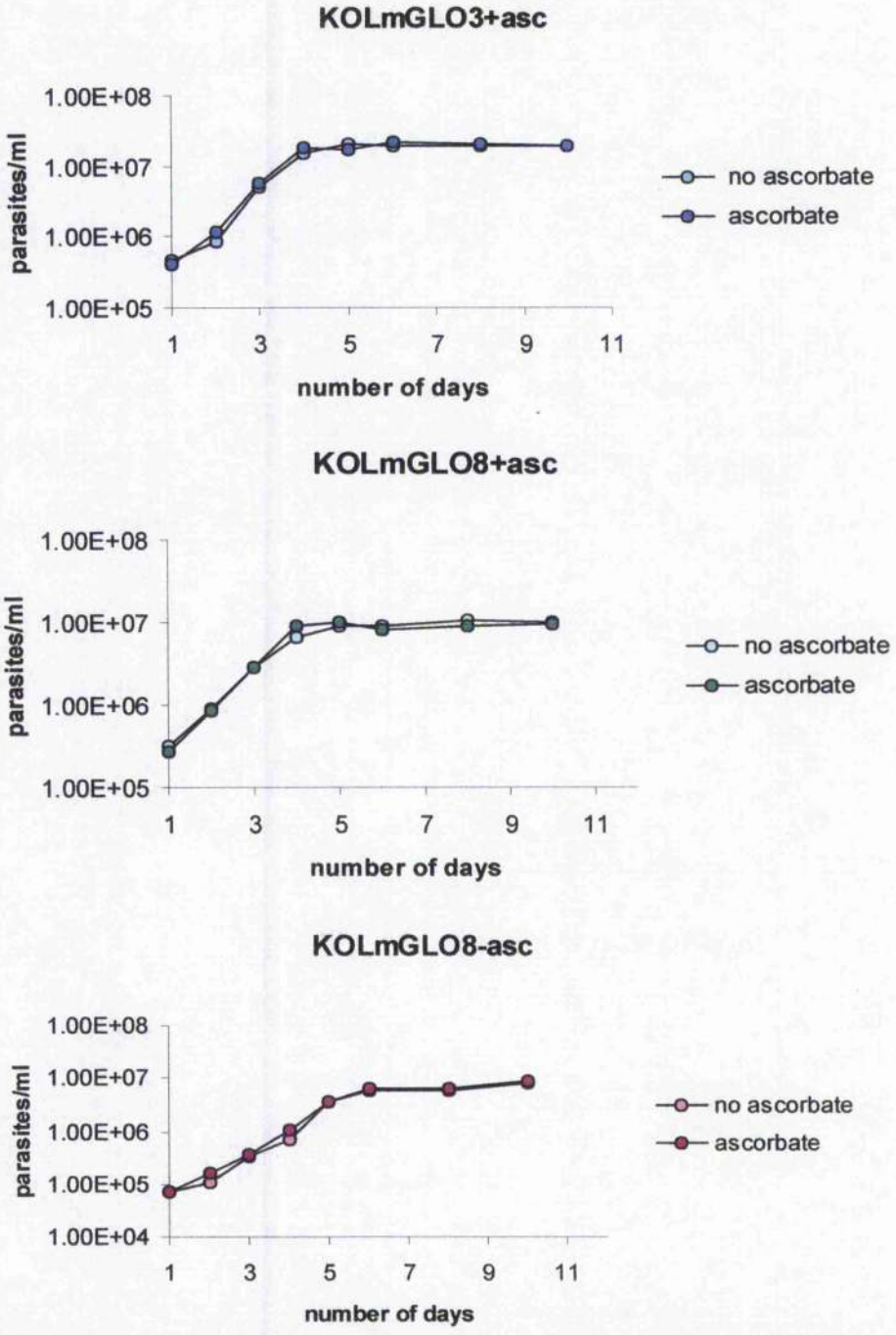

Figure 6.14: Growth curves showing KOLmGLO3+asc, KOLmGLO8+asc and KOLmGLO8-asc with and without exogenous ascorbate. Cultures were seeded at a concentration of 2 x 10^5 parasites/ml and were counted daily. All lines were lines were grown with 15 µg/ml blasticidin and 100 µg/ml hygromycin. Ascorbate was added to cultures as indicated to a final concentration of 10 mM. Exogenous ascorbate had no effect on the growth rates of these *L. major* lines.

6.2.6.2 *In vitro* infectivity to macrophages

The ability of KOLmGLO3 and KOLmGLO8 stationary phase promastigote parasite lines to infect peritoneal macrophages was assessed (figure 6.15). Both these lines had previously been grown with ascorbate, as described. KOLmGLO3 infected a similar percentage of macrophages as WT parasites: approximately 20%. However KOLmGLO8 parasites infected a lower number of cells: approximately just 5%. Once again, the difference in phenotype is observed in the parent line of the clone found to be genuine LmGLO knockouts. Therefore the loss of LmGLO may also cause reduced infectivity in *L. major in vitro*. However, despite the variation in infection levels observed between the WT and KOLmGLO8 lines, the difference was not found to be significant. This is likely to be due to the high standard errors and the low number of replicates performed and further investigation is required to show conclusively that the LmGLO knockout parasites are defective in their ability to infect macrophages.

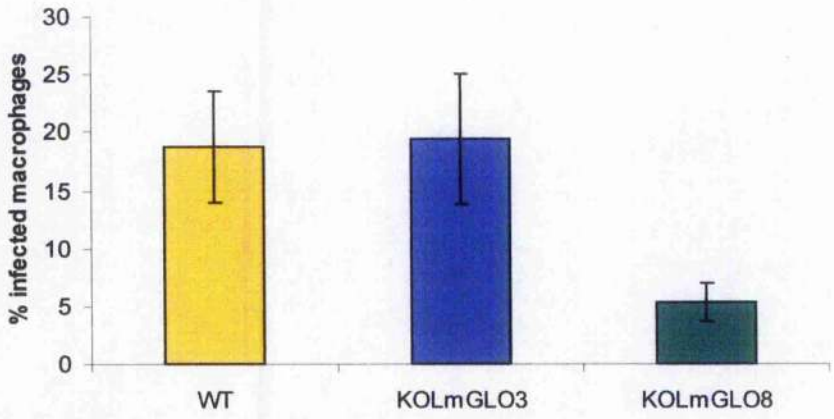

Figure 6.15: Infectivity of WT, KOLmGLO3 and KOLmGLO8 *L. major* promastigotes to macrophages. Stationary phase promastigotes were used to infect peritoneal macrophages at a rate of 2:1 and the slides were incubated for 5 days post-infection. Results are the means ± SE from at least two experiments. The WT and KOLmGLO8 lines did not infect the macrophages at significantly different rates ($p > 0.05$).

6.2.6.3 *In vivo* infectivity to mice

In vivo infectivity of the clones KOLmGLOA1 and KOLmGLOB were assessed. Parasites in the stationary phase of growth were inoculated into mouse footpads, the thicknesses of which were then measured over time (figure 6.16). Relative to the infections caused by WT parasites, infections caused by KOLmGLOB were significantly much less rapid in growth, as determined by the increase in footpad thicknesses. After five weeks the footpads infected with WT parasites had increased from an average of 1.8 mm to 4.3 mm while those infected with KOLmGLOB parasites had reached an average of just 2.1 mm. Similar results were obtained with KOLmGLOA1 parasites. After 10 weeks the KOLmGLOB infected footpads were on average 4 mm thick while those infected with KOLmGLOA1 were on average just 2.5 mm although did continue to increase thereafter. So both clones caused attenuated infections to occur as compared to WT parasites. Given that KOLmGLOB was shown to lack LmGLO while KOLmGLOA1 is unlikely to, this was an unexpected result. In order to grow in the presence of the selective drugs, the KOLmGLOA1 parasites must either be supporting one or more extra-chromosomal plasmids or have one or more constructs, intended for integration at the LmGLO locus, integrated elsewhere in their genome. It is reasonable to consider that either of these scenarios could be detrimental to the parasites, explaining their decreased infectivity to mice, but that the reduced infectivity of the KOLmGLOB parasites may be due to the loss of the LmGLO gene. However, it is also possible that both clones were damaged during the transformation procedure, hence the similar phenotype observed.

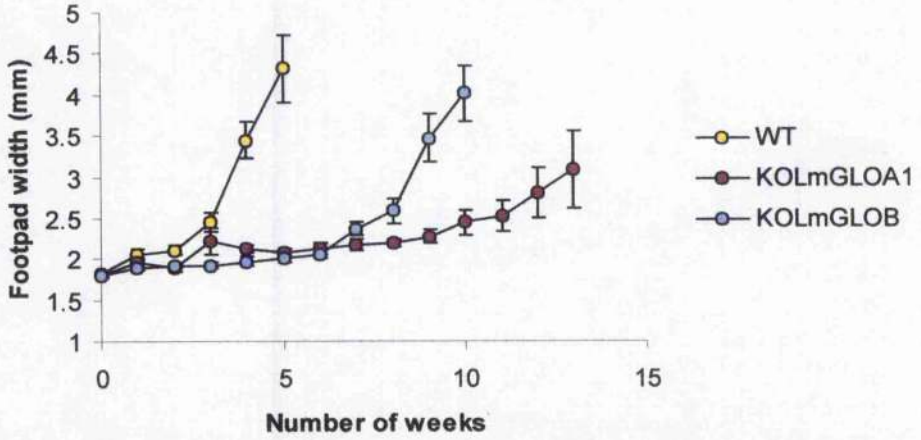

Figure 6.16: Infectivity of WT, KOLmGLOA1 and KOLmGLOB *L. major* promastigotes to mice. 5 x 10^5 stationary-phase promastigotes were resuspended in 20 µl PBS and inoculated into one footpad of each BALB/C mouse. The footpad thicknesses were subsequently measured weekly, and the results are the means ± SD from five mice. The footpad thicknesses of mice infected with WT parasites were significantly greater than those of mice infected with KOLmGLOB five weeks post-infection ($p < 0.0004$).

6.3 Discussion

Ascorbate synthesis was originally of interest due to the ability of TDR1 to reduce oxidised ascorbate *in vitro*. Whether TDR1 also functions as a dehydroascorbate reductase in *Leishmania* remains controversial. As detailed in the introduction to this chapter, reduction of dehydroascorbate is thought to occur non-enzymatically by trypanothione (Krauth-Siegel and Ludemann, 1996) as parasite lysates failed to increase ascorbate being formed in *in vitro* experiments. However, in order to minimise spontaneous reaction the assay was performed at pH 6.5 although the optimal pH for DHAR activity *in vitro* has previously been reported to be ~8.0 (Maellaro *et al.*, 1997; Girardini *et al.*, 2002). Therefore the pH may have prevented GSH-dependant DHAR activity being detected in the assay; the absence of a positive control being performed means this cannot be ruled out. Trypanosomatids do not possess glutathione reductase, which, as detailed in section 6.1.4, is important for dehydroascorbate reduction by glutathione-dependant enzymes, as it in turn recycles glutathione. As trypanosomatids instead possess trypanothione reductase, it may be the case that trypanothione-dependant proteins would be responsible for any enzymatically mediated recycling of ascorbate. This would be analogous to *Leishmania* containing trypanothione S-transferases rather than glutathione S-transferases, as has recently been shown (Vickers *et al.*, 2004). However, glutathione can be reduced by trypanothione and glutathione-dependant enzymes do prevail in trypanosomatids (Wilkinson *et al.*, 2002b). Whether any enzymatic DHAR present in *T. cruzi* could have been dependant on trypanothione rather than glutathione has not been investigated.

The recently published account of TbALO (Wilkinson *et al.*, 2005), an ALO-like *T. brucei* enzyme capable of converting L-galactono-1,4-lactone to ascorbate and D-arabinono-1,4-lactone to erythroascorbate, to an extent addresses the initial questions postulated here of whether trypanosomatids were capable of synthesising ascorbate and whether it was important for viability. LmGLO shares 45% identity with TbALO although the region between amino acid 235 and amino acid 320 poorly aligns and is of very low similarity. It may be significant that LmGLO lacks the C-terminal SHL motif thought to target the *T. brucei* protein to the glycosome as differences in localisation of ascorbate synthesis between the parasite species could be indicative of diverse roles for ascorbate.

TbALO is so called because it is most like yeast ALO in terms of substrate specificity although investigation into the TbALO amino acid sequence revealed that, like LmGLO, it is most similar to mammalian GLOs. However TbALO was unable to utilise the mammalian substrate L-gulono-1,4-lactone, therefore diminishing the likelihood of the

mammalian synthesis pathway operating in trypanosomatids. Indeed the authors of the TbALO paper propose that it is the plant L-galactose pathway that is most likely to be responsible for ascorbate synthesis in *T. brucei*, based on unnamed sequences that they have identified in the genome. Several sequences similar to those of plant ascorbate synthesis enzymes were identified in *Leishmania* (section 6.2.1). Due to the role of GDP-6-mannose pyrophosphorylase in a range of cellular processes, the presence in *Leishmania* of the similar sequence LmjF23.0110 may not be relevant to ascorbate synthesis and as such has not been considered here.

Conversely, the activity of the plant L-galactose-1-phosphate phosphatase protein is thought to be exclusive to the ascorbate synthesis pathway and therefore the discovery of the similar *Leishmania* sequence LmjF17.1390 was intriguing. However, analysis of the mouse – and indeed human- protein databases revealed that they too possess similar sequences to L-galactose-1-phosphate phosphatase which were not more similar to any other *A. thaliana* protein. Clearly experimental characterisation is required to ascertain whether these mammalian proteins are involved in a different cellular process or whether they too have a role in ascorbate synthesis. For example it is feasible that they could dephosphorylate D-glucuronic acid-1-phosphate (figure 6.1). The existence of the mammalian sequences certainly cast doubt on whether LmjF17.1390 is indeed a dedicated ascorbate synthesis protein exclusive to the plant L-galactose pathway. A set of proteins similar to yeast and plant enzymes that mediate the penultimate ascorbate synthesis steps - arabinose dehydrogenase, L-galactose dehydrogenase and galacturonic reductase were - also identified in *Leishmania*. However, these were all more similar to other yeast and plant proteins and one of them – LmjF31.2150 – has been experimentally characterised as a prostaglandin f2 alpha synthase (Kabututu *et al.*, 2003). Meanwhile LmjF28.1230, which is similar to the mammalian aldono-lactonase which mediates the penultimate step in ascorbate synthesis in mouse, is more similar to the aldono-lactonase than any other mouse protein. Therefore the substrate specificity of TbALO is the only evidence of the plant pathway being operational in trypanosomatids. Controversy already exists over whether plant GALDH can utilise L-gulono-1,4-lactone as a substrate; *A. thaliana* cells used it as a precursor for ascorbate synthesis (Wolucka and Van Montagu, 2003) while purified sweet potato GALDH could only metabolise L-galactono-1,4-lactone (Oba *et al.*, 1995). It is possible that activity with L-gulono-1,4-lactone is difficult to reproduce *in vitro*. It is clear that experimental characterisations of several enzymes - including LmGLO and the putative ascorbate synthesis proteins identified and described in this chapter - are required before any conclusions regarding which pathway(s) is responsible for ascorbate

biosynthesis in the trypanosomatids. In addition the potential for *Leishmania* to scavenge different intermediates in the synthesis pathways should be addressed.

Indeed, the ability to scavenge ascorbate is the reason postulated to explain the exacerbated growth defect of *T. brucei* TbALO null mutants in ascorbate-free media compared to normal media (Wilkinson *et al.*, 2005). Serum is reported as having an ascorbate concentration of 40 μM but it is not stated what percentage of the final media is comprised by serum. Standard media is often 10% (v/v) serum, which would give a final concentration of 4 μM ascorbate, much lower than the reported Kms for known ascorbate and dehydroascorbate transporters (Rumsey *et al.*, 1997; Daruwala *et al.*, 1999; Rumsey *et al.*, 2000). Although not described in detail here, searches of the *L. major* genome for possible transporters were conducted using enquiry sequences of known ascorbate transporters (as detailed in section 6.1.3). There were no significantly similar proteins to the sodium-dependant transporters SVCT1 and SVCT2, or to the *E. coli* sgaT transporter. There was, however, a similar protein to the GLUT transporters, which was most like GLUT3. The GLUT transporters are only known to transport oxidised ascorbate (dehydroascorbate), and the method used by Wilkinson *et al.*, to eliminate ascorbate from the medium was to treat it with ascorbate oxidase, meaning that only dehydroascorbate would remain. Depleting the ascorbate in this way contributed to the growth defects of the TbALO knockout mutants, suggesting that they could accumulate ascorbate but not dehydroascorbate. Therefore, if trypanosomatids are able to uptake ascorbate from their surrounding environment, it is unlikely to be via GLUT-like transporters and may occur by a novel mechanism.

Ascorbate was added to a final concentration of 10 mM to *L. major* parasites that had just undergone the second transfection to knockout the LmGLO gene. This at first seemed to be beneficial as cultures that contained the added ascorbate grew better than those without. However there was no noticeable effect on growth when ascorbate was withdrawn from the cultures that had previously contained it, or added to the cultures that were previously without it. There was also no correlation between the presence of ascorbate in the media and successful LmGLO knockouts: all three clones were derived from cultures that had contained ascorbate and of these only one, KOLmGLOB, proved to be a genuine LmGLO knockout.

The parent strain of KOLmGLOB exhibited several defects: promastigotes grew slowly in culture, did not reach high densities at the stationary phase of growth and were poorly infective to peritoneal macrophages. Moreover KOLmGLOB parasites were poorly

infective to mice, taking several weeks longer than WT parasites to cause lesions of 4 mm to develop. The inability to synthesise ascorbate in these lines appears to adversely affect the parasites although further experimentation is required to address why the KOLmGLOA1 clone also exhibits an *in vivo* infection defect. A more in-depth phenotypic analysis of the clones rather than the parent knockout lines, together with the creation and analysis of KOLmGLO parasites engineered to re-express LmGLO, will help clarify the role of GLO in *L. major*. It is also of interest that parasites over-expressing LmGLO appear to be more infective to both macrophages and mice than WT and control parasites. However, until a method can be developed to prove that the parasites do indeed over-express LmGLO, these results must be treated with caution.

7 Discussion

This study aimed to identify and characterise a *Leishmania* protein capable of reducing pentavalent antimonial compounds to the toxic trivalent form, and to ascertain whether any protein identified was involved in antimonial susceptibility *in vivo*. *L. major* TDR1 was found and investigated due to its similarity to oGST and like this mammalian protein, recombinant TDR1 was capable of reducing pentavalent metalloids, including antimonial drugs, *in vitro* (Denton *et al.*, 2004). To elucidate the role of TDR1 in antimonial metabolism *in vivo*, *L. major* TDR1 knockout and over-expressing lines were generated, infected into macrophages and treated with pentavalent antimonials, and the effects of the drugs on the parasites were assessed. Due to the different macrophage infection rates displayed by the different parasites lines it proved difficult to compare the effects of the pentavalent antimonials on the different parasites. However despite being inconclusive, the studies suggested that altering the level of TDR1 in *L. major* had little or no effect on the parasites' sensitivity to pentavalent antimonial drugs.

If the TDR1 does not have an impact upon the susceptibility of *L. major* to pentavalent antimonials *in vivo* it is interesting to postulate why that may be. Since it is already well established that toxicity is dependant on reduction of the drugs to the trivalent form, the most obvious explanation is that *in vivo* the protein does not reduce pentavalent antimonials and there could be several reasons for this. Firstly, as discussed in the introductory chapter to this investigation, the site of reduction remains controversial. If reduction occurs in the macrophage, which could be mediated enzymatically by one of the classes of mammalian arsenate reductases discussed or by an as yet unidentified protein, TDR1 may only encounter the trivalent form of the drug. It has also been demonstrated that pentavalent antimonials are reduced upon reaction with small thiols such as glutathione and cysteine (Frezard *et al.*, 2001; Ferreira Cdos *et al.*, 2003); this could also occur in the macrophage. Indeed the acidic environment of the phagolysosome where the parasites reside promotes this reaction (Alexander and Russell, 1992). Reduction could also be mediated in the macrophage by a secreted *Leishmania* protein and indeed, it was shown as part of this investigation (chapter four) that TDR1 is likely to be released. This could also be the case for the recently identified *Leishmania* ACR2 protein which reduces pentavalent antimonials (Zhou *et al.*, 2004): although the published account of this protein describes it as comprising 130 amino acids it is possible that it contains a further 100 amino acid N-terminal region which includes a signalling peptide that is predicted to direct the protein to the extracellular space, as outlined in chapter three. In this situation the

macrophage or parasitophorous vacuole could act as a reservoir for the secreted parasite protein meaning that although a *Leishmania* protein would mediate the reaction, reduction and therefore toxicity would be dependant on the amastigote parasites being situated in macrophages. This could help explain some of the controversy surrounding the site of reduction, specifically the observation that axenic amastigotes are not susceptible to pentavalent antimonials (Sereno *et al.*, 1998). However, conflicting reports that axenic amastigotes are indeed sensitive to these drugs (Ephros *et al.*, 1999) imply that the presence of macrophages is not necessary for reduction to occur, and that therefore this happens within the parasites.

The second reason that would explain TDR1 not reducing pentavalent antimonials *in vivo* is that the drugs may be reduced by a different route inside the parasite. Given that there is scant information on the localisation of antimonials within *Leishmania*, it may be that TDR1 and the pentavalent drugs do not co-localise *in vivo*, therefore explaining why the protein is not able to reduce the compounds. This would be particularly likely if the pentavalent antimonials were sequestered inside a parasite organelle given the likely cytosolic distribution of TDR1 described in chapter four. In this case, pentavalent antimonials must be reduced by an alternative mechanism. Again, this could be mediated by a protein other than TDR1 – such as LmACR2 – or could occur non-enzymatically upon reaction with thiols. As well as pentavalent antimonials being reduced by the thiols mentioned, the trypanosomatid-specific thiol trypanothione is also capable of reducing antimonials non-enzymatically (Ferreira Cdos *et al.*, 2003). Indeed, reduction rates were greater in the presence of trypanothione than glutathione, which may go some way to explain why pentavalent antimonials are more toxic to parasites than the host. However, the rates of reduction of antimonials achieved with thiols are a fraction of those achieved with TDR1 *in vitro* (Denton *et al.*, 2004) so non-enzymatic, thiol-based reduction alone is unlikely to account for why TDR1 does not reduce the drugs in vivo. Therefore enzymatic reduction by another *Leishmania* protein or several proteins may be a more likely explanation. As well as LmACR2, other arsenate reductase-like sequences are present in the parasite that may be capable of reducing antimonials, the most alike being similar to GAPDH as explained in chapter three. However, the *in vivo* relevance of GAPDH in metalloid reduction remains unclear. Three new classes of proteins with arsenate reductase activity have been identified in the last five years and it is likely more will be elucidated. A candidate could be the glutaredoxins, of which there are predicted to be several of in *L. major*. Despite the low-level sequence similarity that the proteins share, oGST's and glutaredoxins exhibit comparable activities *in vitro* including thioltransferase and dehydroascorbate reductase activities. It is therefore pertinent to consider that the

glutaredoxins may display the pentavalent arsenical reducing ability of oGST: there are no published accounts of these activities being looked for in glutaredoxins. It would be interesting to look for this activity in the predicted *L. major* glutaredoxins that are described in chapter three.

As well as the possibility that TDR1 does not reduce pentavalent antimonials *in vivo*, it is possible that the protein does indeed carry out this reaction but that the experiments described in this investigation failed to detect this. Although knockout and over-expressing lines are useful tools that have been used to analyse the roles of other *Leishmania* proteins, the approach may not have been successful here. If several proteins, including TDR1, are able to reduce the drugs *in vivo* then knocking out or over-expressing TDR1 may not result in a big difference in the parasites' ability to reduce the pentavalent compounds and therefore render any changes in susceptibility to the drugs undetectable. This effect would be particularly pronounced if the *L. major* parasites were able to up- or down-regulate the expression of these proteins in response to the altered level of TDR1. As discussed in chapter three, *L. major* parasites are thought to possess several glutaredoxins and thioredoxins and degeneracy between these proteins has been reported in yeast (Potamitou *et al.*, 2002): it could be the case that degeneracy between these proteins and TDR1 occurs in *Leishmania* given the common activities of the proteins *in vitro*. It would be interesting to test this hypothesis by comparing the levels of glutaredoxins and thioredoxins in *Leishmania* wild type with the lines with altered TDR1 levels. This could be achieved by individually by western blotting if the specific anti-sera to these proteins were available, or by a more global proteomic approach.

There are other reasons why the approach used to elucidate the role of TDR1 in antimonial reduction and sensitivity *in vivo* may have failed. One possibility could be that the peritoneal macrophages used in the experiments may have been unsuitable for assaying the effects of the drug, for example if there expression profile was altered due to their artificial growth conditions leaving them unable to accumulate the pentavalent antimonials. This would also explain the lack of sensitivity to the drugs displayed by all the parasite lines used in the study. A problem of this nature could be circumvented by infecting live animals with the parasite lines and testing the efficacy of the drugs in treating the *L. major* infections in a true *in vivo* setting. Another potential problem could be that *L. major* parasites that were used in this study: *L. major* causes cutaneous leishmaniasis whereas visceral leishmaniasis, commonly caused by *L. donovani* or *L. infantum*, is more likely to be treated with pentavalent antimonials. Indeed, several studies have shown that intrinsic differences in species sensitivity to these drugs is common and cutaneous leishmaniasis

isolates are generally less susceptible to these drugs than those causing visceral disease (reviewed in Croft *et al.*, 2006). Creating and analysing TDR1 knockout and over-expressing forms of a different species of *Leishmania*, such as *L. infantum*, may be worthwhile.

Very little is known about the enzyme(s) that mediate the reduction of pentavalent metalloids in mammals. There are three classes of enzymes known to display this activity *in vitro* – GAPDH, oGSTs and PNP – and yet evidence of the involvement of any of these proteins *in vivo* remains elusive. Construction of a knockout mouse model of any of these proteins may facilitate this area of investigation and help clarify whether the protein does mediate reduction *in vivo*, although this may be impossible for GAPDH and PNP due to their ubiquity in cells. A greater understanding of mammalian reduction of metalloids is required before the impact, if any, of the host metabolism on the sensitivity of *Leishmania* to pentavalent antimonials can be assessed. It is important to identify the proteins that regulate the reduction of pentavalent metalloids in higher eukaryotes for reasons other than their potential involvement in leishmaniasis chemotherapy: Arsenical compounds continue to contaminate drinking water in many parts of the world and are considered to be a serious health risk. It is distressing to think that how the body metabolises and detoxifies these compounds and how they exert their toxicity is still unknown. With arsenical- and antimonial-based drugs being proposed for the treatment of cancer (Murgo, 2001; Wyllie and Fairlamb, 2006), it is even more imperative that a greater insight into their metabolism is reached. Elucidation of how pentavalent metalloids enter cells – both mammalian and parasitic – is also required.

The recent licensing of the oral antileishmanial drug miltefosine together with the impressive cure rates quoted in several clinical studies of the drug may have prompted speculation that the days of antimonials in leishmaniasis chemotherapy are numbered. However this is a controversial view. It was outlined in the introduction to this investigation that miltefosine, despite its obvious advantages of being orally administrated, has several potential shortcomings: resistance to the drug has been easily generated in *Leishmania* in the laboratory; it may not be effective in treating some South American *Leishmania* species; patients co-infected with HIV tend to relapse when treated with miltefosine; and the drug is teratogenic. Although resistance to pentavalent antimonials is now a problem in some parts of India, these drugs have been used for decades successfully. Although miltefosine is no doubt a welcome addition to the arsenal of antileishmanial drugs, it has not stood the test of time. This, coupled with the infrastructure needed to treat leishmaniasis sufferers with pentavalent antimonials already being in place, means that

antimonials are very much still required and that research into its reduction and activation are valid and important. This research may also aid the understanding of additional metalloid-based chemotherapies.

What the endogenous role is of TDR1 in the parasite is an interesting point. Unlike its *T. cruzi* homologue Tc52, TDR1 was knocked out of *L. major* with relative ease showing that is it is not an essential protein. This is an intriguing difference between the two proteins. The different predicted active sites between TDR1 and Tc52 could be indicative of differing functions which may explain why Tc52 is essential while TDR1 is not. Whatever the reason, the fact that *L. major* can survive well without the protein implies that, as discussed, degeneracy between proteins may be occurring. The *in vitro* activities of TDR1 coupled with the observation that the TDR1 knockout lines were more susceptible to paraquat stress suggest that the protein has a role in protecting the parasite from oxidative stress. Maintaining the correct redox environment is of utmost importance in the cell and this is particularly true of parasites that have to withstand high levels of reactive oxygen species (ROS) generated during the oxidative burst. It would therefore make sense that the parasites have evolved to possess several proteins that are capable of regulating levels of ROS, and that partial degeneracy exists between them.

One of the potential roles of TDR1 in *L. major* is to maintain levels of ascorbate by reducing dehydroascorbate which occurs *in vitro*. This is controversial: it has been described how trypanothione alone is thought to be sufficient for performing this function and that an enzyme is not required (Krauth-Siegel and Ludemann, 1996). However, this observed activity prompted us to look for enzymes that could have a role in ascorbate biosynthesis in *L. major* and the discovery of LmGLO which, as explained in chapter six, is likely to have such a role. The potential involvement of TDR1 in reducing ascorbate remains unresolved although it would be interesting to analyse this. For example, in the TDR1 knockout lines LmGLO could be up-regulated: if the ability to recycle ascorbate was lost or reduced, a solution could be to produce more ascorbate. The reciprocal is true: in the LmGLO line TDR1 could be up-regulated to promote recycling of any available ascorbate. This hypothesis could be tested by western blotting. Furthermore, it would be of interest to attempt to generate a double mutant lacking both proteins: if the hypothesis was correct this mutant may not be viable. Certainly, what both these proteins do have in common is that directly or indirectly they are likely to impact upon the intracellular redox environment of the parasite.

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