Studies on Oligopeptidase B of *Leishmania major*

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Abstract

Peptidases of *Leishmania* are acknowledged virulence factors. It is hypothesised that peptidases are crucial for the survival of *Leishmania* in its hosts and that many could be potential targets for new antileishmanial drugs. As such, the investigation of peptidase activity in live *Leishmania* promastigotes was proposed as a valuable approach by which to increase knowledge on particular peptidases. In order to complete this investigation, it was decided to use short peptidyl fluorogenic substrates, which only fluoresce once the bond linking the peptide to the fluorescent moiety is cleaved. These allow detection of peptidase activity by quantifying the release of the fluorescent moiety.

Detection of peptidase activity in live *Leishmania* using the fluorogenic substrate Bz-R-AMC proved fruitful, enabling study of the activity of the serine peptidase oligopeptidase B (OPB) in live *L. major* promastigotes. OPB is a member of the Family S9 peptidases, the prolyl-oligopeptidases, which are taxonomically restricted to plants, bacteria and trypanosomatid flagellates. In African and American trypanosomes, OPB has been shown to have important roles: OPB is a virulence factor in *Trypanosoma cruzi*, mediating entry into host cells, and OPB is released into the serum by African trypanosomes, where it cleaves host blood factors.

In this study, the inhibition profile of *L. major* OPB has been determined and OPB has been localised to the cytosol, the site of hydrolysis of Bz-R-AMC. Immunoprecipitation of OPB confirmed that OPB was the sole peptidase responsible for the hydrolysis of Bz-R-AMC and anti-OPB antibodies were found to inhibit the hydrolysis of Bz-R-AMC. Inhibitors of OPB could also kill *Leishmania* promastigotes, suggesting OPB could be a valuable drug target.

However, genetic manipulation of OPB was successful, with mutants over-expressing OPB and Δ*opb* null mutants produced. OPB is thus not essential for the growth of promastigote *L. major*, though the Δ*opb* null mutants did have a defect in metacyclogenesis, in survival in macrophages and a reduced ability to induce lesions on the footpads of mice. A role in amastigote differentiation or survival in macrophages was also suggested. OPB is thus likely to be a virulence factor, though not essential, and thus not suitable as a primary drug target.

A number of avenues require further investigation, including the need for re-expression of OPB in the Δ*opb* null mutants to confirm that lack of OPB is indeed responsible for the phenotypic deficiencies of the null mutants. Other important areas requiring attention are investigation of the role of OPB in amastigote differentiation or survival, investigation of the reported release of OPB by promastigotes, and identification of the physiological substrate of OPB.

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Author's Declaration

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

Jane Claire Munday
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List of Abbreviations

AA	axenic amastigotes
AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride
AMC	7-amino-4-methylcoumarin
ANF	atrial natriuretic factor
ATP	adenosine triphosphate
BA*p*NA	*N*-α-benzoyl-L-arginine-*p*-nitroanilide
bp	base pair
Bz	benzoyl
CFU	colony forming units
CL	cutaneous leishmaniasis
CP	cysteine peptidase
CPA	cysteine peptidase A
CPB	cysteine peptidase B
CPC	cysteine peptidase C
CPRG	chlorophenol red β-D-galactopyranoside
CR1 / 3	complement receptor type 1 / type 3
Δ*opb*	null for *OPB* gene
DAPI	4,6-diamidino-2-phenylindole
DC	dendritic cell
DCI	3,4-dichloroisocoumarin
DFP	diisopropyl phosphofluoridate
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
E-64	*trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane
E-64d	(2S,3S)-*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester
EDTA	ethylenediamine tetra acetic acid
ED_{50}	effective dose to kill 50%
EF-1α	elongation factor 1α from *T. brucei*
GFP	green fluorescent protein
GPI	glycoinositol-phospholipid
h	hour
HEK cells	human embryonic kidney cells
HIFCS	heat inactivated foetal calf serum
HRP	horseradish peroxidase
HYG	hygromycin B
IC_{50}	inhibitory concentration for 50% inhibition
IFN-α	interferon α
IFN-γ	interferon γ
IL	interleukin
iNOS	inducible nitrogen oxide synthaseαα
K11777	N-methyl-piperazine-Phe-homoPhe-vinylsulfone-phenyl
kb	kilo base
kDa	kilo dalton
K_i	inhibition constant; binding efficiency of inhibitor
KO	knock out
l	litre
LaOPB	OPB from *L. amazonensis*
LB	Luria-Bertani
LD_{50}	lethal dose to kill 50%

L. inf.	*Leishmania infantum*
L. maj.	*Leishmania major*
L. mex.	*Leishmania mexicana*
LPG	lipophosphoglycan
LUC	ectopic luciferase-expressing *Leishmania*
µ	micro
m	milli / metre
M	molar
min	minute
MHC	major histocompatibility complex
MMP	matrix metalloprotease
MØ	macrophage
ms	millisec
MVT	multivesicular tubule
n	nano
nd	not done
NO	nitric oxide
OD	optical density
OPB	oligopeptidase B
PBS	phosphate buffered saline
PCMB	*p*-chloromercuribenzoic acid
PCR	polymerase chain reaction
pefabloc	see AEBSF
PEM	peritoneal exudate macrophage
PG	phosphoglycan
P.I.S.	pre-immune serum
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear neutrophil granulocytes
PMSF	phenylmethylsulfonyl fluoride
POP	prolyl oligopeptidase
PPG	proteophosphoglycan
PSG	promastigote secretory gel
PV	parasitophorus vacoule
sec	second
SAT	streptothricin acetyltransferase
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
sp	subpassage number
Suc	3-carboxy-propionyl
TbOPB	OPB from *T. brucei*
TcOPB	OPB from *T. cruzi*
TcgOPB	OPB from *T. congolense*
TeOPB	OPB from *T. evansi*
Th	T helper cell
TLCK	*N*-tosyl-L-lysyl-chloromethylketone
TNF-α	tumour necrosis factor α
TGF-β	transforming growth factor β
TPCK	*N*-tosyl-L-phenylalanyl-chloromethylketone
TSE	trypomastigote soluble extract
U	unit
VL	visceral leishmaniasis
WHO	World Health Organisation
WT	wild type
Z	benzoyloxycarbonyl

Chapter 1

INTRODUCTION

1.1 *Leishmania*

1.1.1 Causative agent of leishmaniasis

Leishmania are obligate intracellular protozoa of the order Kinetoplastida and family Trypanosomatidae. They are the causative agent of leishmaniasis, which is endemic in various tropical and subtropical regions (Herwaldt, 1999a). Around 20 species of *Leishmania* are able to infect humans and 30 phlebotomine sand fly species have been identified as vectors (Desjeux, 2004). Leishmaniasis is a complex of diseases, which may be classified into four clinical forms:

i) Visceral leishmaniasis (VL), also known as kala azar, causes life-threatening systemic infection. Symptoms include fever, severe weight loss, anaemia, hepato-splenomegaly, pancytopenia and hypergammaglobulinaemia. Most cases of VL are caused by *L. donovani*, particularly in the Indian subcontinent and Eastern Africa. In the Mediterranean and the New World VL is due to *L. infantum* and *L. chagasi*, which are now generally accepted to be one species (Olliaro et al., 2002).

ii) Localised cutaneous leishmaniasis (CL) is the most common form and causes 1-200 skin lesions, generally confined to the bite site. It is caused mainly by *L. major*, *L. tropica* and *L. aethiopica* in the Old World and by *L. mexicana* complex species, *L. guyanensis* and *L. panamensis* in the New World. The lesions usually self heal within a few months, though they can cause disfiguring scars. It can generally be resolved without recourse to drugs (Desjeux, 2004).

iii) Diffuse cutaneous leishmaniasis is a chronic progressive form characterised by disseminated cutaneous lesions, which are non-ulcerative (Herwaldt, 1999a), due to a defective cell-mediated immune response (Desjeux, 2004). The lesions are comparable to those of leprosy and, unlike those of localised CL, do not self-heal. It is caused by *L. aethiopica* in the Old World and *L. mexicana* complex species, particularly *L. amazonensis*, in the New World.

iv) Mucocutaneous leishmaniasis is a metastatic complication of cutaneous leishmaniasis caused by *L. braziliensis* subspecies. Sores develop on the naso-oropharyngeal mucosal membranes with consequent disfigurement (Herwaldt, 1999a).

Leishmaniasis is endemic in 88 countries worldwide, 66 in the Old World and 22 in the New World (Desjeux, 2001), as shown in Figure 1.1. The disease burden is very high, with an estimated prevalence of 12 million people infected and 350 million people at risk. There is substantial under-reporting of the number of cases, but it is estimated that there are 1-1.5 million new cases of CL and 500,000 of VL annually (www.who.int/entity/leishmaniasis). Over 90% of cases of VL occur in Bangladesh, Brazil, India, Nepal and Sudan; 90% of cases of CL occur in Algeria, Afghanistan, Brazil, Iran, Iraq, Peru, Saudi Arabia and Syria (Herwaldt, 1999a).

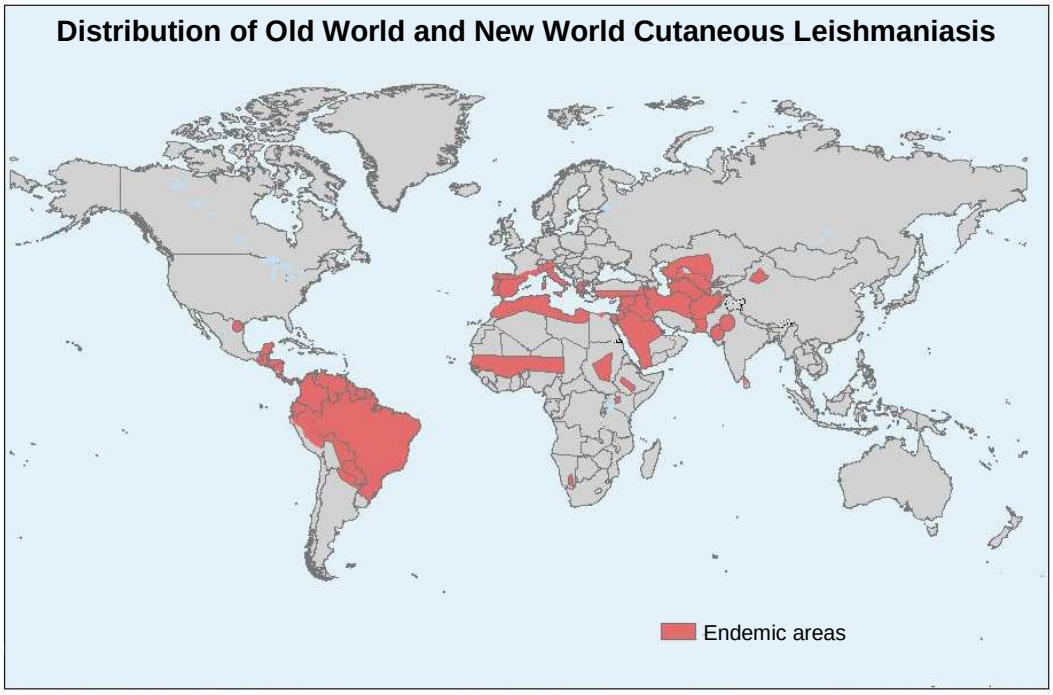
1.1.2 Life cycle

Leishmania alternate between their mammalian host and the digestive tract of female phlebotomine sand flies. Most *Leishmania* species (those of subgenus *Leishmania*) are suprapylarian parasites, restricted to the midgut of the sand fly, but the New World *Viannia* subgenus parasites are peripylarian parasites, entering the hindgut before moving forward to the midgut (Kamhawi, 2006).

When a sand fly bites an infected mammal it takes up a bloodmeal containing *Leishmania* amastigotes. These amastigotes must differentiate into promastigotes to survive in the sand fly. Six stages of promastigotes have been described, which *Leishmania* differentiate into sequentially as they move anterially through the midgut (Rogers et al., 2002). Amastigotes transform into procyclic promastigotes, which then develop into nectomonad promastigotes, then leptomonad promastigotes and finally metacyclic promastigotes, which are the stage infective to mammals. There are also haptomonad promastigotes and paramastigotes, both of which have been found only in low numbers, and for neither of which stage has the precursor form been definitely determined (Rogers et al., 2002; Kamhawi, 2006). Only two of the six stages identified have been shown to be actively replicative: i) procyclic promastigotes, in the abdominal midgut, the bloodmeal phase, and ii) leptomonad promastigotes, in the thoracic midgut and foregut, the sugarmeal stage (Gossage et al., 2003). This life cycle is illustrated in Figure 1.2.

The development of *Leishmania* within the sand fly, from amastigote to infective metacyclic promastigotes, has been found to take 6-9 days, depending on the species (Kamhawi, 2006). Metacyclic promastigotes accumulate at the stomodeal valve for transmission. The thoracic midgut is filled by promastigote secretory gel (PSG) which contains mostly leptomonad and metacyclic promastigotes and which

A



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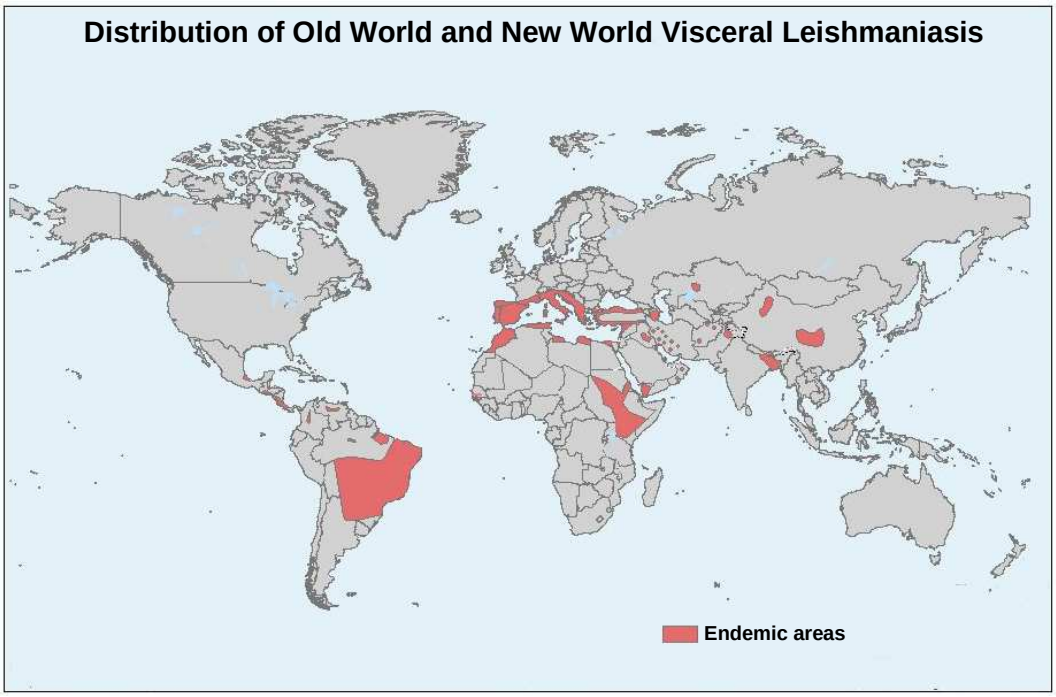
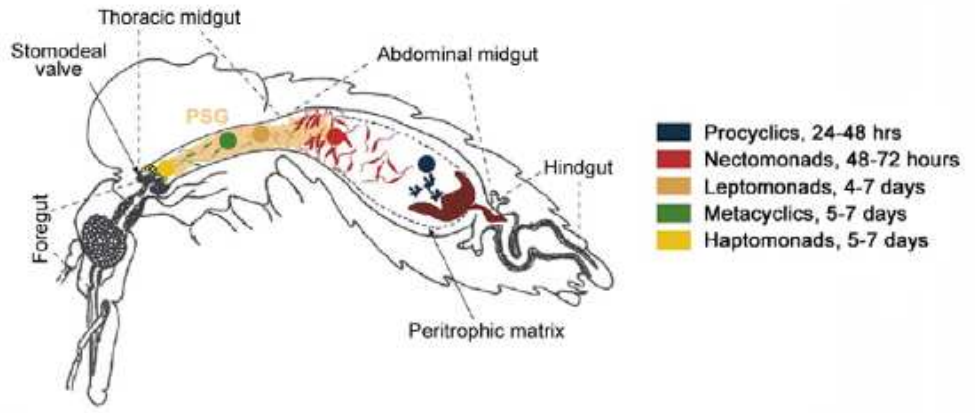


Figure 1.1 Distribution of cutaneous and visceral leishmaniasis worldwide.
A = cutaneous leishmaniasis, B = visceral leishmaniasis.
Maps from World Health Organisation (www.who.int/leishmaniasis/leishmaniasis_maps).

A



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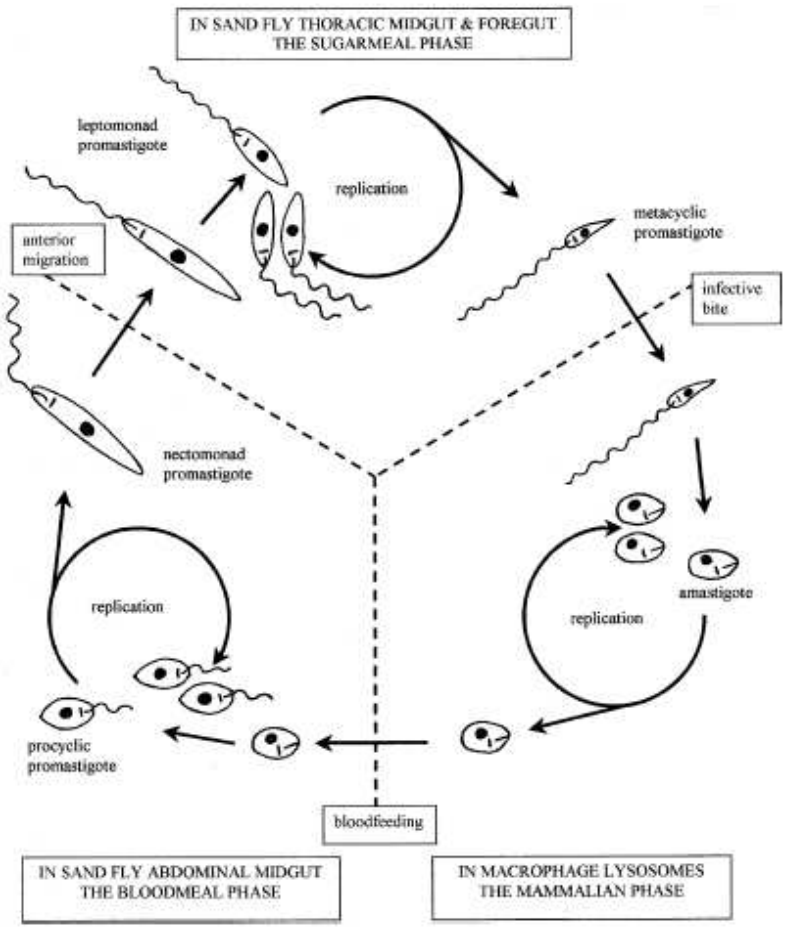


Figure 1.2 Life cycle of *Leishmania* in sand fly.
A = schematic representation of a *Leishmania* infected phlebotomine sand fly, illustrating time-dependant appearance of distinct morphological forms of promastigotes within the midgut. Thoracic midgut is filled with promastigote secretory gel (PSG) and the stomodeal valve is held open by this PSG. (From Kamhawi, 2006).
B = digenic life cycle of *Leishmania*. Parasite replication only occurs at three points: i) amastigotes in the parasitophorous vacuole of macrophages, ii) procyclic promastigotes in the sand fly abdominal midgut and iii) leptomonad promastigotes in the sand fly thoracic midgut. The growth phases are linked by various non-dividing stages as is shown. (From Gossage et al., 2003).

blocks the midgut, forcing open the stomodeal valve (Rogers et al., 2002). The presence of the PSG plug and a heavy infection with *Leishmania* is associated with a reduced ability to take in blood, numerous attempts to feed and thus more chances for transmission. It has been found that around 1000 metacyclic promastigotes are regurgitated into the mammalian host when a sand fly feeds, with most coming from behind the stomodeal valve (Rogers et al., 2004). The PSG plug has also been shown to be regurgitated into the mammalian host and it has been found that the main component of this, filamentous proteophospho-glycan (fPPG), enhances disease progression (Rogers et al., 2004).

When metacyclic promastigotes are injected into the mammalian host polymorphonuclear neutrophil granulocytes (PMN) are the first cells recruited to the bite site, appearing within 10-24 hours post infection (Muller et al., 2001). *L. major* promastigotes were found to be chemotactic for neutrophils, attracting them to the bite site (van Zandbergen et al., 2004). *Leishmania* promastigotes are phagocytosed by PMN and inhibit spontaneous apoptosis of the PMN, prolonging the cell lifespan until the point when macrophages are recruited to the bite site, normally within 1-2 days after infection. Macrophages then phagocytose the infected PMN (Aga et al., 2002). This has been suggested to be a “Trojan horse” by which the parasite can infect the macrophage silently, without activating the antimicrobial functions of the macrophage (Laskay et al., 2003). PMN can be infected by promastigote *Leishmania*, but the parasites stay as this form: they do not transform into an amastigote, the mammalian replicative stage of the parasite (van Zandbergen et al., 2004), nor multiply (Laskay et al., 2003), until they are within the parasitophorous vacuole (PV) in a macrophage. Amastigotes are aflagellate and round-oval in shape with a diameter of approximately 4 µm (Herwaldt, 1999a). Amastigotes reside within PVs within the macrophage and these vacuoles differ depending on the species of *Leishmania*: they can be tightly or loosely fitted around the amastigotes, and one vacuole can harbour a single amastigote or multiple amastigotes (Rittig and Bogdan, 2000). For example, *L. mexicana* and *L. amazonensis* PVs are large and contain many amastigotes, whilst *L. major*, *L. infantum* and *L. donovani* produce only small vacuoles which fit closely around the amastigotes (Handman and Bullen, 2002), as illustrated in Figure 1.3. The amastigotes multiply within the PV and are released to infect further cells. The mechanism of the release is not fully characterised. The macrophage was presumed to burst due to the physical pressure of large numbers

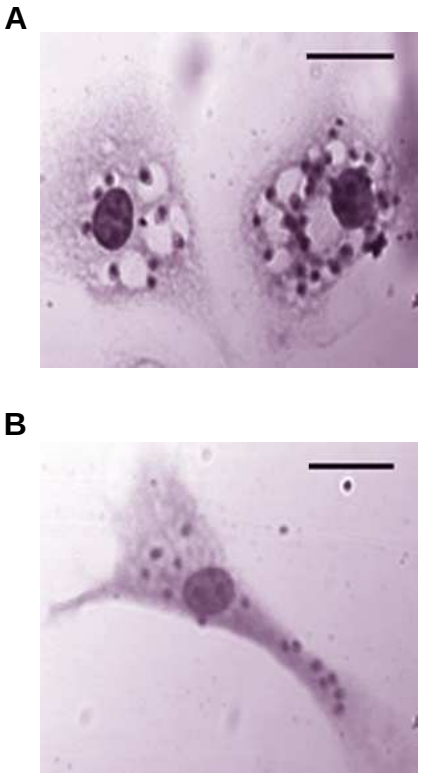


Figure 1.3 Amastigotes within parasitophorous vacuoles in mouse peritoneal exudate macrophages. A = PEM infected with *L. mexicana*, B = PEM infected with *L. infantum*. Infected PEM were stained with Giemsa's stain. False colour image. Scale = 10 µm.

of amastigotes, but recent evidence suggests that the parasite may induce the exocytosis machinery of the macrophage to enable release (Rittig and Bogdan, 2000).

1.1.3 Ultrastructure of *Leishmania*

The ultrastructure of *Leishmania* differs from that of other kinetoplastids and also shows variation between the different life cycle stages of the parasite (Clayton et al., 1995). The various forms of the *Leishmania* life cycle were initially characterised according to their dimensions, flagellar morphology, kinetoplast (mitochondrial DNA) position and the relative distance from the nucleus to the basal body of the flagellum (Clayton et al., 1995). The characteristic shapes of the stages are maintained by an array of cross-linked subpellicular microtubules underneath the plasma membrane (McConville et al., 2002).

The ultrastructure of the promastigote and amastigote stages of *Leishmania* is illustrated in Figure 1.4. Promastigotes are motile cells with long thin bodies, of size up to 5 µm by 20 µm, with a flagellum up to 20 µm, whilst amastigotes are non-motile ovoid cells of approximately 4 µm diameter with only a remnant flagellum.

1.1.3.1 Specialised organelles

Leishmania contain many of the organelles found in higher eukaryotes, but they also contain a number of special organelles.

The surface membrane of *Leishmania* is covered in a distinct glycocalyx made of glycolipids. This glycocalyx is thick on promastigote stages and thin on amastigotes and is thought to enable survival of the promastigote in the sand fly (Turco et al., 2001). The main surface molecule is the hyperglycosylated form of glycoinositol-phospholipid (GPI), lipophosphoglycan (LPG). GPI-linked proteins, including the GPI-linked surface metallopeptidase GP63, and proteophospho-glycans (PPGs) are also present on the surface of *Leishmania* (McConville et al., 2002). The role of LPG in virulence in mammalian hosts is dependant on the species. LPG is essential for the survival of *L. major* and *L. donovani* promastigotes in the sand fly (Sacks et al., 2000) and for *L. major* infection of macrophages and mice (Spath et al., 2000). However, *L. mexicana* parasites

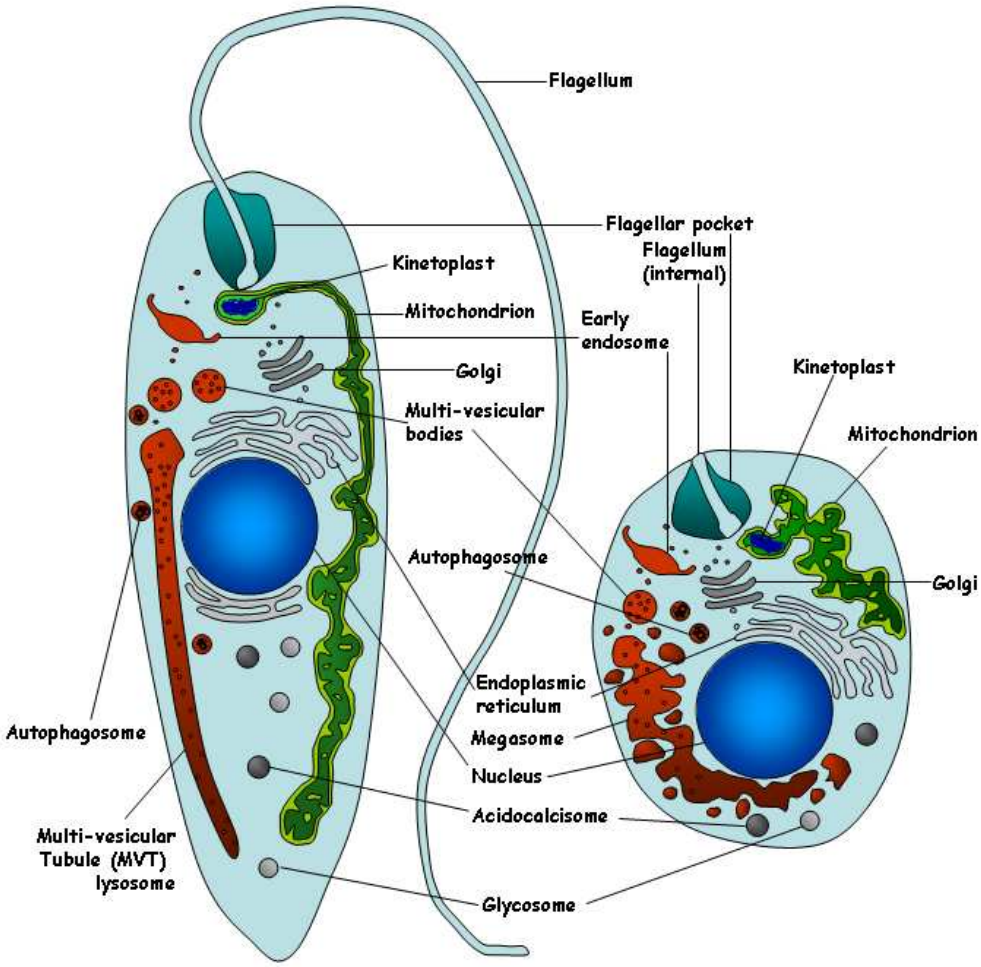


Figure 1.4 Schematic representation of the main organelles of *Leishmania* in both promastigote and amastigote form. Promastigote on the left and amastigote on the right. The flagellar pocket is at the anterior end of the cell. Taken from Besteiro et al., 2007.

lacking LPG are still infective to macrophages and mice (Ilg et al., 2001). The role of all surface molecules containing phosphoglycan (PG) has also been investigated in *L. major*, with a lack of PGs on promastigotes confirmed to lead to a defect in macrophage infectivity. However, there was no effect on the infectivity of amastigotes (Capul et al., 2007). This is probably due to amastigotes having a much reduced glycocalyx, lacking GPI-anchored macromolecules, having instead a surface layer of free GPIs and host-derived glycophospholipids (McConville et al., 2002).

Both promastigotes and amastigotes secrete a large quantity of soluble PPG. These form, individually or after self association, large filamentous structures. PPG structures may lead to promastigote aggregation and the transmission of large clusters of promastigotes in the sand fly bite (McConville et al., 2002), with PPG known to be the main component of the PSG plug in the sand fly, which, as has been stated earlier, is regurgitated into the mammalian host and enhances disease progression (Rogers et al., 2004).

The subpellicular microtubules below the cell membrane form the cytoskeleton and restrict vesicular transport to the only region of the cell not subtended by the microtubules: the flagellar pocket at the anterior end of the cell (Clayton et al., 1995). The flagellar pocket is an invagination of the plasma membrane, with the junction between the cell membrane and the flagellum being formed of desmosome-like tight junctions (de Souza, 2002). The flagellar pocket is the single location for endo- and exo-cytosis, for integration of membrane proteins into the cell membrane, and is accessible to large macromolecules (Landfear and Ignatushchenko, 2001), for example secreted LPG and PPG, and endocytosed MHC class II molecules (De Souza Leao et al., 1995).

The flagellum contains a classical 9 + 2 microtubule doublet axoneme as well as the trypanosomatid-specific paraflagellar rod (Bastin et al., 2000). The flagellum is thought to have a number of functions. It is responsible for the motility of promastigote *Leishmania*, and is known to mediate attachment of *Leishmania* to the sand fly gut epithelium (Killick-Kendrick et al., 1974a; Killick-Kendrick et al., 1974b). It is also postulated to act as an environmental sensor and to be involved in signalling (Bastin et al., 2000). The amastigote stage has a much shortened flagellum which does not emerge from the flagellar pocket, making this stage immotile.

The flagellum of *Leishmania* is anchored into the cell by a basal body, which is physically linked to the mitochondrion. A second basal body, lying perpendicular to the major one, develops during cell division. In *T. brucei*, the basal bodies are necessary for kinetoplast division (Ogbadoyi et al., 2003), whilst in *L. donovani* duplication and segregation of kinetoplasts have been observed despite a lack of basal body duplication (Selvapandiyan et al., 2004).

The kinetoplast is a disc shaped structure, comprising the condensed mitochondrial DNA (Liu et al., 2005). The kinetoplast DNA (kDNA) represents 10-20% of the total cell DNA and is composed of a few dozen maxicircles and thousands of minicircles (Stuart, 1983). The maxicircles have some of the functions of higher eukaryote mitochondrial DNA, encoding genes for the respiratory complexes. The minicircles are responsible for the network structure of kDNA (Stuart, 1983) and encode guide RNAs which mediate the editing of the maxicircle transcripts to form functional mRNA, by insertion of uridylate residues to precise sites (Liu et al., 2005). In *T. brucei*, the kinetoplast is attached to the mitochondrial membrane by unilateral filaments and to the basal body via exclusion zone filaments. These filaments constitute the tripartite attachment complex (TAC), which may mediate interactions between the mitochondrion and the cytoskeleton (Ogbadoyi et al., 2003). It has been postulated that the TAC is present in *Leishmania* and the other trypanosomatids (Ogbadoyi et al., 2003), though this has not been confirmed.

In *Leishmania* there is a single mitochondrion which extends throughout the cell. In dividing promastigotes the mitochondrion is a symmetrical organelle, extending from both sides of the kinetoplast region and joined at the posterior end of the cell. In contrast, in non-dividing promastigotes the mitochondrion is asymmetric, extending from one end of the kinetoplast past the nucleus to the cell posterior (Simpson and Kretzer, 1997), as shown in Figure 1.4. In amastigotes the mitochondrion is in the form of a complex network extending throughout the cell (Coombs et al., 1986). In addition to the mitochondrion, *Leishmania* produce energy within specialised organelles called glycosomes, which contain the glycolytic enzymes responsible for converting glucose to 3-phosphoglycerate (de Souza, 2002). Glycosomes are small, spherical organelles, belonging to the peroxisome family (Michels et al., 2006). *Leishmania* also contain acidocalcisomes, spherical organelles preferentially located at the periphery of the

cell, which are believed to be storage organelles for a number of elements including phosphorus, calcium, magnesium, sodium and zinc (de Souza, 2002).

As with other eukaryotes, *Leishmania* contain a single Golgi apparatus, located between the nucleus and the flagellar pocket and consisting of a stack of 3 to 10 cisternae and a polymorphic *trans*-Golgi network. The endoplasmic reticulum (ER) consists of the nuclear envelope and a system of cisternae that are often closely associated with the plasma membrane (McConville et al., 2002). The ER provides some of the membranes necessary for the biosynthesis of endosomes and lysosomes. In promastigotes the endocytic pathway has been shown to consist of three parts: i) a network of tubular endosomes close to the flagellar pocket, ii) a population of multivesicular bodies also in the flagellar pocket region of the cell, and iii) an unusual multivesicular tubule (MVT) running along the anterior-posterior axis of the cell (Waller and McConville, 2002). The MVT is the terminal compartment of the endocytic pathway, forming the mature lysosome and so is known as the MVT-lysosome (Ghedin et al., 2001; Mullin et al., 2001; McConville et al., 2002). The lytic capacity of the MVT increases as the promastigotes reach the stationary phase of growth (Mullin et al., 2001). In *Leishmania* amastigotes there is an extended lysosomal compartment comprising one or more large vesicles that have been termed megasomes, which were originally found only in *L. mexicana* group species (Pupkis et al., 1986; Ueda-Nakamura et al., 2001), but have recently been identified in *L. chagasi* amastigotes (Alberio et al., 2004). Megasomes make up 5-15% of the amastigote cell volume depending on the species (Alberio et al., 2004). The large increase in lysosomal capacity with the appearance of megasomes in amastigotes coincides with the large increase in expression of cysteine peptidases resident in these cells (Waller and McConville, 2002). In addition to the cysteine peptidases, megasomes also contain a number of other enzymes, including arylsulfatase, β-glucuronidase, DNAse and RNAse (Pupkis et al., 1986).

The lysosome of *Leishmania* is postulated to have a number of functions. For example, it may be involved in nutrient acquisition, evasion of the immune system and regulating the levels of endogenous proteins (Waller and McConville, 2002). Lesion amastigotes have been found to degrade host macromolecules (Schaible et al., 1999) and to influence the immune system by degrading host MHC class II complexes, thus governing the range of peptides which can associate with MHC molecules.

1.1.4 Treatment of leishmaniasis

The drugs currently available for treating leishmaniasis are the pentavalent antimonials, i.e. sodium stibogluconate (Pentostam) and meglumine antimonate (Glucantime); amphotericin B and its lipid formulations; pentamidine; miltefosine and paromomycin (Croft and Coombs, 2003). All current drugs have their limitations, with all except miltefosine requiring parenteral administration, necessitating long hospitalisation. In addition, the drugs available are not equally effective against all types of leishmaniasis.

1.1.4.1 Visceral leishmaniasis

For visceral leishmaniasis, the pentavalent antimonials have been the first line treatment for over 60 years (Croft and Coombs, 2003). Antimonials are toxic drugs, giving rise to musculoskeletal pain and gastrointestinal side effects, with potential for serious adverse effects including cardiac arrhythmia and pancreatitis. High levels of resistance to these drugs have been reported in endemic regions, particularly around the focus of infection in Bihar, India (Sundar and Rai, 2002). Due to its toxicity, pentamidine has generally only been used as a second line therapy following ineffective antimonial treatment. Treatment failures with pentamidine have also been reported in India (Sundar and Rai, 2002). Amphotericin B is highly effective at treating antimony-resistant cases, but is also toxic, with infusion related fever and chills, nephrotoxicity and first-dose anaphylaxis reported. It has, however, replaced antimonials as a first line treatment in some areas of Bihar (Chappuis et al., 2007). The lipid formulation, AmBisome, has much reduced toxicity, but its high cost has limited its use in developing countries until recently (Croft and Coombs, 2003). The WHO announced a price reduction in May 2007 for AmBisome in VL-endemic countries, which reduced treatment cost from around $2,800 to $200 (Chappuis et al., 2007).

Miltefosine was licensed for use in India in 2002 (Davies et al., 2003). It is an alkyl phosphocholine which was originally designed as an anti-cancer drug and was found to have anti-leishmanial activity against *L. donovani* and *L. infantum* in a mouse model (Croft et al., 1987; Kuhlencord et al., 1992; Croft et al., 1996). Miltefosine has been shown to be safe for both adults and children in India and although gastrointestinal side effects are common, these do not generally prevent completion of treatment (Sundar et al., 2002; Sundar et al., 2003). However, it

was found to be a possible teratogen in animal studies (Herwaldt, 1999b), so its use in women of child-bearing age has to be carefully monitored (Sundar et al., 2002). Miltefosine has the advantage of being orally administered, but it has a long half life in the body, which may influence the development of resistance (Guerin et al., 2002), and such resistance has been generated in a laboratory setting (Seifert et al., 2003; Perez-Victoria et al., 2006; Seifert et al., 2007). In addition, it has not been established whether miltefosine will be as effective against *L. infantum/chagasi* as it is against *L. donovani* (Croft and Coombs, 2003).

Paromomycin, an aminoglycoside antibiotic, was identified as an anti-leishmanial in the 1960's, but was only licensed for use in India in 2006 following development co-ordinated by WHO/TDR (www.who.int/tdr). Paromomycin was found to be as effective as amphotericin B when trialled (Sundar et al., 2007). It has drawbacks: it has to be administered by intramuscular injection, it causes reversible damage to the inner ear in 2% of patients, and it may produce mild injection pain (Chappuis et al., 2007), but it does have the major advantage of being low cost, as it is out of patent .

1.1.4.2 Cutaneous leishmaniasis

Since cutaneous leishmaniasis often self-heals, drug treatment is not always necessary, and is normally only used if there is a risk of mucocutaneous leishmaniasis. For patients with relapsing or disfiguring lesions or diffuse or mucocutaneous leishmaniasis, systemic treatment with pentavalent antimonials is most commonly used (Davies et al., 2003). Treatment failures have been documented for New World cutaneous leishmaniasis (Lawn et al., 2003) and it is known that there is a difference in the susceptibility of different *Leishmania* species to pentavalent antimonials; *L. donovani* and *L. braziliensis* are more sensitive compared to *L. major*, *L. tropica* and *L. mexicana* (Croft et al., 2002). Pentamidine is successfully used to treat cutaneous and mucocutaneous leishmaniasis (Sundar and Rai, 2002), and amphotericin B, particularly liposomal amphotericin B, is often used as a first line treatment for travellers and as a second line treatment after relapse (Schwartz et al., 2006).

Miltefosine is currently undergoing clinical testing against New World species of *Leishmania*. It is effective against *L. panamensis*, less so against *L. mexicana*, and shows a large variation in its efficacy against *L. braziliensis* (Soto et al., 2004;

Soto et al., 2007). Miltefosine has also been tested against Old World cutaneous species and found to give similar cure levels as antimonials (Mohebali et al., 2007). Topical treatment with paromomycin has been found to be effective against both Old and New World species (Schwartz et al., 2006; Mohebali et al., 2007), with ointments of paromomycin combined with methylbenzethonium having increased effectiveness compared to those combined with urea (Arana et al., 2001).

1.1.4.3 Effect of immunosuppression

Increased levels of HIV prevalence in *Leishmania*-endemic countries has led to increasing numbers of co-infected patients, with 34 countries currently reporting co-infection (www.who.int/leishmaniasis). HIV-*Leishmania* co-infection leads to worse outcomes for the patient for both diseases. Both HIV-co-infected patients and immunosuppressed patients respond slowly to treatment and in most cases relapses occur (Olliaro et al., 2002). Non-antimonial treatment is thought to be preferred in immunocompromised patients: AmBisome and miltefosine have been shown to be effective in mouse models of VL-infected immunodeficient mice, where antimonial treatment was ineffective (Escobar et al., 2001). Amphotericin B has been shown to be more effective than pentavalent antimonial treatment in HIV co-infected patients (Sundar and Rai, 2002), with antimonials also associated with pancreatitis in immunosuppressed patients (Bryceson, 2001). Co-infected or immunosuppressed patients are also believed to be likely to develop resistance to anti-leishmanial drugs if given in monotherapy, a resistance which could then spread throughout the community, meaning combination therapy is desirable (Bryceson, 2001).

1.1.4.4 Future treatment options

New treatments for leishmaniasis are needed, but development is hampered by the lack of funds available for costly drug development for what is seen as mostly a developing world disease (Guerin et al., 2002).

A number of potential anti-leishmanial drugs have been identified and are at various stages of development. Those furthest through development are sitamaquine and the azoles. Sitamaquine is an 8-aminoquinoline which was identified by the Walter Reid Army Institute of Research, but is being developed by GlaxoSmithKline. Sitamaquine is orally administered and has shown some degree

of success in Phase I/II trials against visceral leishmaniasis in Brazil and Kenya (Croft and Coombs, 2003), but further toxicological testing is underway after unanticipated nephrotoxicity was found in trials. The azoles were discovered as antifungal drugs and several, including ketoconazole, itraconazole and fluconazole, have entered clinical trials for both cutaneous and visceral leishmaniasis (Singh et al., 2006). They inhibit the formation of ergosterol, which is the major sterol in *Leishmania*, compared to cholesterol in the mammalian cell (Urbina, 1997). There are suggestions from clinical trials that they are more effective against *L. major* and *L. mexicana* than *L. donovani* and *L. braziliensis* (Croft et al., 2002).

Research into potential drugs continues, with a more rational approach to drug design being taken (Davis et al., 2004). Fundamental research into *Leishmania*-specific drug targets is one of the most promising areas. Through a rational approach to identifying drug targets, several have already been proposed and investigated, including cysteine peptidases, the metabolism of trypanothione and sterol biosynthesis enzymes and protein kinases (Croft & Coombs, 2003). However, the recent availability of the genomes of three *Leishmania* species and powerful bioinformatic tools has identified *Leishmania*-specific genes, making them attractive potential drug targets (Peacock et al., 2007), and will hopefully lead to identification of the parasite factors responsible for disease tropism and pathology of the disease which may be good potential targets (Ivens et al., 2005). As early branching eukaryotes, the organisation of their cells show extensive differences from that of mammalian cells (as described in Section 1.1.3), suggesting that there will be ample basis for identifying novel proteins, structures and processes that may be exploitable as drug targets (Barrett et al., 1999).

1.1.5 Potential for vaccination

Vaccination has been a focus of research for many years, since it is known that patients who have recovered naturally from cutaneous infection maintain residual parasites indefinitely, but are resistant to reinfection (Handman, 2001). This suggests that vaccination is a viable prospect for preventing infections. A form of vaccination, leishmanisation, was practised for decades in the Middle East and Soviet Union (Selvapandiyan et al., 2006). This was controlled infection, inducing lesions on unexposed areas of skin, in order to prevent future lesions on visible skin, particularly the face. The procedure holds risks, however, with the potential for large, non-healing lesions and even immunosuppression, and has been

discontinued (Handman, 2001; Chappuis et al., 2007). Killed *Leishmania* vaccines have also been investigated, but no preparations of killed parasites have shown protective efficacy (Chappuis et al., 2007).

Latterly, strategies using live-attenuated parasites, recombinant DNA, peptides, purified parasite proteins and non-protein antigens have been investigated (Handman, 2001; Selvapandiyan et al., 2006). It is hoped that the recent creation of a promising multi-epitope subunit vaccine will lead to progress, since it has shown efficacy against *L. major* and *L. infantum* infections in animal models (Skeiky et al., 2002; Coler et al., 2007) and its efficacy is being tested in human trials (Coler and Reed, 2005). There is also an increasing number of genetically altered parasites which are being tested as candidate live attenuated vaccines (Alexander et al., 1998; Papadopoulou et al., 2002; Uzonna et al., 2004), although their efficacy in humans has yet to be investigated.

1.2 Interaction Between *Leishmania* and Host Cells

1.2.1 Invasion of macrophages

The entry of *Leishmania* into macrophages is thought to involve both silent entry through the “Trojan Horse” of infected neutrophils, as described in Section 1.1.2, and direct entry into macrophages. The binding of *Leishmania* promastigotes to macrophages is thought to be primarily through the complement receptors type 1 (CR1) and type 3 (CR3), which bind to complement components attached to molecules on the parasite plasma membrane (Antoine et al., 1998). The CR1 binds the C3b fragment of complement and CR3 binds iC3b, with the latter thought to be the more important (Mosser and Rosenthal, 1993). Bound iC3b on the parasite surface prevents complement lysis, with GP63 on the parasite surface being responsible for rapid conversion of C3b to iC3b (Cunningham, 2002; Denkers and Butcher, 2005). Amastigote binding to macrophages is not dependant on serum and involves different receptors than binding of promastigotes (Mosser and Rosenthal, 1993).

Leishmania are thought to be taken up by conventional phagocytosis, with elongated pseudopodia advancing tunnel-like along the parasites (Rittig and Bogdan, 2000), into a phagosome which then merges with endosomes and lysosomes to form a phagolysosome (Bogdan and Rollinghoff, 1999). When

promastigotes are phagocytosed, LPG on the parasite surface inhibits phagosome-endosome fusion (Desjardins and Descoteaux, 1997; Dermine et al., 2000). Since promastigotes are acid labile but amastigotes are not (Bogdan and Rollinghoff, 1999), delaying the development of this organelle promotes survival, allowing promastigote-amastigote differentiation to be completed (Bogdan and Rollinghoff, 1999). Amastigote survival in the acidic PV is thought to be due to the protective effects of the abundance of cell surface and secreted glycoconjugates produced by this stage (Sacks and Sher, 2002).

The phagolysosome has features of lysosomal compartments, being positive for the lysosomal-associated membrane proteins LAMP-1 and LAMP-2, having acidic pH and containing acidic hydrolases normally found in lysosomes (Russell et al., 1992). They are seen as being a mixed organelle, also having features of late endosomes, since they contain the late-endosome associated proteins rab7p and macrosialin (Lang et al., 1994; Courret et al., 2002). Fusion with endocytic organelles is believed to occur in a sequential manner, with endosomes fusing prior to lysosomes (Duclos and Desjardins, 2000). A “kiss and run” fusion hypothesis has been proposed for the biogenesis of phagosomes, with organelles joining their plasma membranes to form a fusion pore, which allows exchange of molecules between organelles; the pore then closes and the organelles separate (Duclos and Desjardins, 2000). This process would allow gradual acquisition of molecules and size selection based on the size of the pore.

1.2.2 Survival of *Leishmania*

In order to survive within macrophages *Leishmania* must achieve silent infection, avoiding activating the anti-microbial respiratory burst and evading immune responses (Bogdan and Rollinghoff, 1999). Induction of a Th1 immune response is associated with clearance of *Leishmania* infection, whilst a Th2 response leads to persistence. The regulatory cytokines TGF-β and IL-10 have also been suggested as important as immunosuppressive signalling molecules in *Leishmania* infections (Olivier et al., 2005). The release of both cytokines has been found to be induced by infection of macrophages by *Leishmania* and mice constitutively-expressing IL-10 cannot control *Leishmania* infection (Olivier et al., 2005). *Leishmania* have also been shown to inhibit production of the Th1 response-promoting cytokines IL-1, IL-12 and TNF-α (Bogdan and Rollinghoff, 1999; Olivier

et al., 2005). Recent *In vivo* studies using *L. major* and *L. donovani* have found that pro-inflammatory cytokines were actually induced in early infection, with the induction being higher in *L. major* infections, suggesting one possible reason for the differences in pathology associated with the two species (Olivier et al., 2005). An analysis of the genes expressed by macrophages following infection by *L. chagasi* has also revealed a general trend for genes encoding Th1-type responses to be down-modulated, whilst genes involved in an anti-inflammatory, Th2-type response were upregulated (Rodriguez et al., 2004). *Leishmania* thus modulate the immune system by both down-regulating Th1 response and up-regulating Th2 responses.

The primary receptor used for uptake of *Leishmania* into macrophages is thought to be protective for the parasite, as uptake of complement opsonised *L. major* via CR3 leads to inhibition of IL-12 release, while uptake via other receptors induces IL-12 release (Schonlau et al., 2000). Uptake of *L. major* or *L. mexicana* amastigotes also suppresses IL-12 production, though this is not dependent on opsonisation by complement (Weinheber et al., 1998). IL-12 is a Th1 response-initiating cytokine which activates natural killer (NK) cells, which in turn produce interferon (IFN)-γ which activates macrophages and is important for early control of infection (McMahon-Pratt and Alexander, 2004).

Binding of complement by *L. major* and *L. donovani* has also been found to increase parasite survival by leading to a reduction of the respiratory burst of macrophages when complement-opsonised promastigotes were phagocytosed (Mosser and Rosenthal, 1993). IFN-γ induces macrophages to produce inducible nitrogen oxide synthase (iNOS) and nitric oxide (NO) production, which are known to be effective at killing *Leishmania* (Olivier et al., 2005). *L. major* have been found to interfere with the induction of iNOS, and purified LPG alone is able to suppress iNOS expression and NO production (Proudfoot et al., 1996). The inhibition of iNOS has been shown to involve abnormal protein kinase C activity (Olivier et al., 2005).

1.2.2.1 Neutrophils in *Leishmania* infection

As well as their role as “Trojan horses” for the infection of macrophages (as detailed in Section 1.1.2), PMN are also important in the survival of *Leishmania* due to their production of cytokines. In mouse models it has been found that neutrophils from mice with a resistant phenotype are associated with parasite

killing, whilst neutrophils from sensitive mice were not. The neutrophils induced different cytokine profiles: TNF-α was induced by neutrophils from resistant mice, whilst TGF-β was induced by neutrophils from sensitive mice (Ribeiro-Gomes et al., 2004). PMN from *L. major*-infected resistant mice have been found to produce not only TNF-α, but also other Th1 response-associated cytokines, particularly IFN-γ, whereas susceptible mice did not (Chen et al., 2005). Depletion of PMN from resistant mice led to a reduction in the expression of Th1 cytokines by macrophages. Depletion of neutrophils in sensitive mice led to an increase in the levels of IL-4, a key Th2 response-promoting cytokine, with an exacerbation of infection (Chen et al., 2005). However, depletion of PMN from sensitive mice has also been found to be beneficial; with impairment in the development of footpad lesions in *L. major* infected BALB/c mice and less IL-4 produced (Tacchini-Cottier et al., 2000). It is suggested that the strains used or the method of depletion of neutrophils used could be responsible for this difference (Chen et al., 2005). In *L. donovani*-infected mice, neutrophils have also been implicated in the protective response against *Leishmania*. Depletion of neutrophils from BALB/c mice led to increased numbers of parasites in the spleen and bone marrow, with an increase of levels of IL-10 and IL-4 in the serum, with impairment of the protective Th1 immune response (McFarlane et al., 2008). The response of neutrophils is therefore implicated as one of the mechanisms of protection against *L. major* (Chen et al., 2005) and *L. donovani* (McFarlane et al., 2008).

1.2.2.2 *Leishmania* and antigen presentation

Leishmania-specific $CD4^+$ T cells have been found to have an essential role in the immune responses of infected mammalian hosts (Antoine et al., 1998). $CD4^+$ T cells recognise peptides bound into MHC class II molecules on the plasma membrane of macrophages. Infection of mouse macrophages by *Leishmania* does not inhibit the induction of MHC class II molecules by IFN-γ, nor does it reduce the number of molecules expressed (Antoine et al., 1998). *L. mexicana*-infected macrophages were found to have MHC class II both on their surface and in the parasitophorous vacuole (Russell et al., 1992). After infection, intracellular MHC class II molecules were redistributed to the periphery of most PVs (Lang et al., 1994), suggesting that the PV is where parasite molecules are processed and loaded for presentation. *Leishmania* have been found to selectively endocytose

MHC class II molecules and to degrade these within their megasomes, due to the cysteine peptidases found in these organelles (De Souza Leao et al., 1995).

Leishmania infections have also been shown to elicit a MHC class I dependent $CD8^+$ T cells response. This was thought to be unlikely since, classically, MHC class I molecules present antigens from the cytoplasm but it has been found that the phagosome, and most likely the PV, can process exogenous antigens for MHC class I molecules (Houde et al., 2003), providing a mechanism for the presentation of *Leishmania* antigens.

1.2.2.3 Manipulation of dendritic cells

Monocyte derived dendritic cells (DCs) are essential to induce a protective Th1 immune response against *Leishmania* (Favali et al., 2007). DCs have been found to be infected with *Leishmania*, though it appears that only amastigotes can successfully invade and persist (Ghosh and Bandyopadhyay, 2004). Langerhans cells (skin DCs) are thought to be a safe haven in early infection with *Leishmania* as, although parasites cannot multiply within them, Langerhans cells can not kill the parasite easily, presumably due to the absence of iNOS in these cells (Bogdan and Rollinghoff, 1998).

DC distribution in *L. donovani* infected mice has been found to be altered, with migration of Langerhans cells reduced by *L. major* LPG, and *in vitro* the mobility of splenic DCs is inhibited by *L. major* secreted products. Alteration of migration of DCs is thought to affect the host immune response, as the DCs normally migrate to lymphoid organs to present antigens to T cells, thereby activating the primary immune response (Ghosh and Bandyopadhyay, 2004).

Different parasite strains appear to have different effects on DCs, principally on the release of IL-12, which promotes the development of a Th1 response. *L. major* strains which had led to self-healing lesions induced IL-12 production, whilst *L. major* strains which had caused multiple subcutaneous nodules in patients and *L. tropica* or *L. donovani* isolates did not induce IL-12 production (McDowell et al., 2002). Reduction of IL-12 may thus be part of the reason for some of the differences seen in the immune response and the pattern of disease (Sacks and Sher, 2002). *L. mexicana* has also been shown to inhibit DC IL-12 production, and to promote production of IL-4 (McMahon-Pratt and Alexander, 2004).

Leishmania can also delay the maturation of DCs and their migration to the lymphoid organs, which is thought to promote establishment of the infection before the acquired immune response can start (Brandonisio et al., 2004). Monocyte-derived DCs exposed to *L. amazonensis* had altered DC differentiation and produced less IFN-γ, making the DCs less effective at inducing adaptive immune responses (Favali et al., 2007).

1.2.3 Nutrient acquisition by *Leishmania*

To survive within their host cell, *Leishmania* must acquire nutrient sources from the macrophage. A number of ligands have been shown to be taken up by endocytosis and delivered to the parasitophorous vacuole (PV) through the endosomal system, with increased efficiency of uptake with increasing age of infection (Russell et al., 1992). The ligands were also detected within the flagellar pocket and megasomes of amastigotes, illustrating that *Leishmania* can take up molecules delivered from the endosomal system.

As well as molecules delivered to the PV via endocytosis, molecules from the host cell cytosol are delivered to the PV. Two mechanisms have been demonstrated whereby molecules can be delivered from the cytosol: firstly via an organic anion transporter on the vacuole membrane, and secondly via the autophagic pathway (Schaible et al., 1999). Autophagy is a self-digestion process whereby cells degrade proteins and organelles in the cytoplasm, by sequestration into a double-membrane vesicle, which normally fuses with the lysosome to allow the degradation of the cargo by hydrolases (Reggiori and Klionsky, 2005). Delivery of molecules to the PV via autophagy may provide nutrients to *Leishmania* in addition to those delivered via fusion with endosomes (Schaible et al., 1999), particularly purines which *Leishmania* are unable to synthesize (Hassan and Coombs, 1988).

Although there is no evidence of other transporters in the PV membrane, a number of other transporters have been identified on lysosomal membranes (Pisoni and Thoene, 1991) and it is likely that these would also be found on the PV membrane. If present, these would allow the movement of monosaccharides, nucleosides, amino acids, sulphate and phosphate from the cytosol into the PV (Burchmore and Barrett, 2001).

The various methods of nutrient acquisition by the PV are illustrated in Figure 1.5.

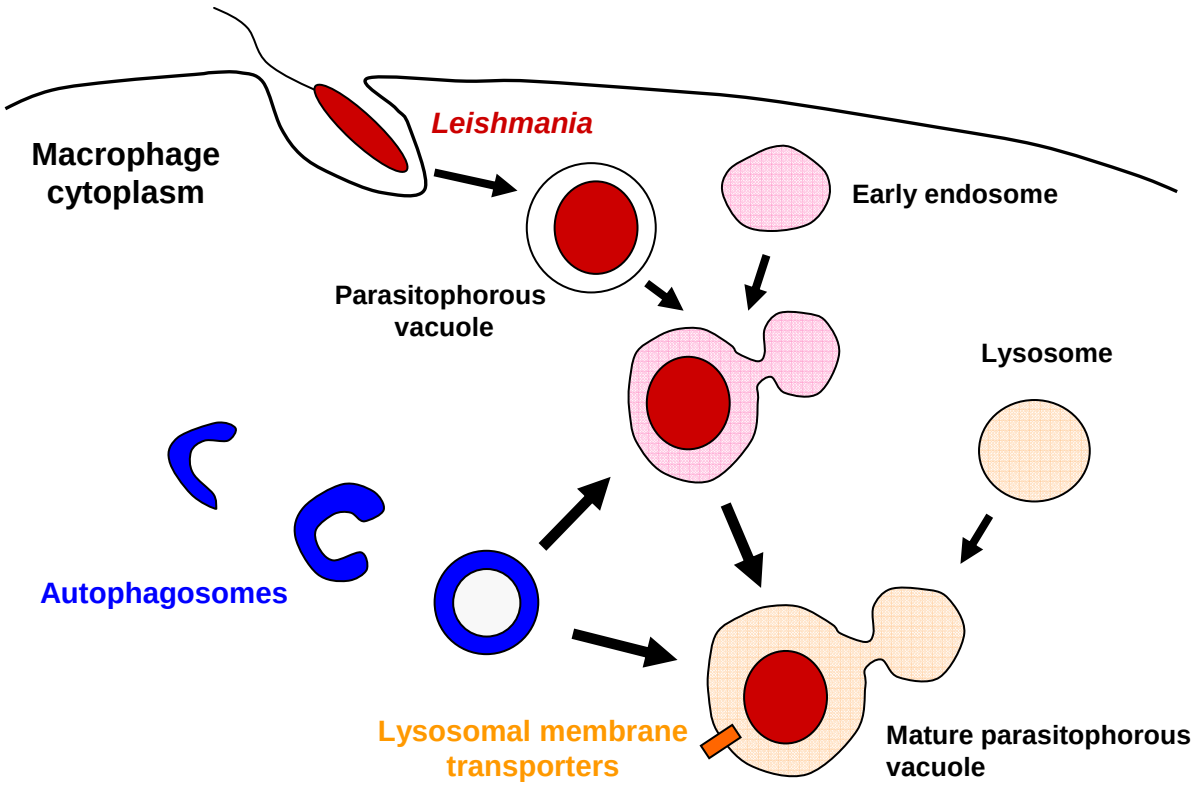


Figure 1.5 Nutrient acquisition by *Leishmania* via interaction with the endosomal and autophagosomal systems of the macrophage and lysosomal membrane transporters. Adapted from Duclos and Desjardins, 2000.

The hydrolases present in the PV ensure that most macromolecules delivered to this organelle are rapidly degraded to low molecular weight solutes (Burchmore and Barrett, 2001). These solutes ought to be ideal nutrient sources for *Leishmania*, with amastigotes being able to use fatty acids, amino acids and glucose as carbon sources (Hart and Coombs, 1982) and amastigotes taking up glucose, proline and polyamines through transporters with acidic pH optima (Burchmore and Barrett, 2001).

A number of proteins characterised as peptidases or involved in proteolysis were detected in an analysis of the *L. donovani* secretome. These enzymes could be involved in degradation of host proteins for the nutrient needs of the parasite. Several *Leishmania* peptidases have been identified as potential virulence factors for survival in the host and this secretion into the PV may be an additional survival mechanism (Silverman et al., 2008).

1.3 Peptidases

1.3.1 Peptidase biochemistry

Peptidases are responsible for the degradation of proteins or oligomeric peptides by hydrolysis of peptide bonds. Enzymes which cleave peptide bonds have previously also been described as proteases or proteinases (Barrett and McDonald, 1986). It was determined in 1936 that there were two types of peptidases, endopeptidases and exopeptidases (Bergmann and Ross, 1936), endopeptidases being those peptidases which cleave bonds within the polypeptide chain, exopeptidases cleaving bonds from the amino or carboxyl ends. Peptidases are numerous and diverse; they represent around 2% of all gene products (Rawlings and Barrett, 1999) and range from monomers of around 10 kDa to multimeric complexes of several hundred kDa (Sajid and McKerrow, 2002).

Although only a single peptide bond is cleaved during a catalytic reaction, the surrounding amino acids in the substrate also play a role in determining peptidase specificity, as do the sequences flanking the catalytic amino acid residues on the peptidase. The convention used to identify substrate and peptidase residues was determined in 1967 by Schechter and Berger, with the substrate residues designated as P and the corresponding residues of the enzyme as S (Schechter and Berger, 1967). Residues are further characterised as prime if they are on the

C-terminal side of the cleavage site and as non-prime if on the N-terminal side. Thus, a five residue substrate cleaved between the third and fourth amino acids would be designated as $P_3P_2P_1$-$P'_1P'_2$.

The naming of peptidases varies from that of other enzymes. The naming of enzymes is usually on the basis of the reactions they catalyse, but the specificities of peptidases are typically almost impossible to rigorously determine or describe in a simple name (Rawlings and Barrett, 1999) and peptidases all effectively catalyse the same reaction: the hydrolysis of a peptide bond (Barrett et al., 2001). Instead, the peptidases were divided into groups on the basis of the catalytic mechanism used (Hartley, 1960), based on the active site residue that provides the nucleophilic attack during the catalytic reaction. Six main types are now recognised: serine, cysteine, metallo-, aspartic, threonine and glutamic peptidases, but there are also a few peptidases of unknown catalytic type. In serine, cysteine and threonine peptidases, the catalytic nucleophile is the reactive group of an amino acid side chain, a hydroxyl group in serine or threonine peptidases or a sulfhydryl group in cysteine peptidases. For aspartic and metallo-peptidases the nucleophile is an activated water molecule. The mechanism of action of glutamic peptidases has not been fully determined (Fujinaga et al., 2004). In the early 1990s, peptidases were classified by both sequence data and crystallographic data in a scheme that brought together sets of peptidases that were similar both in their molecular structure and their evolutionary origin (Rawlings and Barrett, 1993). This classification system was used to create the MEROPS database of peptidases and peptidase inhibitors, using a hierarchical classification system (Rawlings et al., 2008; http://merops.sanger.ac.uk). Related peptidases are grouped into a family, families believed to have had a common ancestor are grouped into clans and the clans are grouped into types based on their active site residue (Rawlings et al., 2008).

1.3.2 Types of peptidases

Most serine peptidases have a catalytic triad of three amino acids: serine, aspartate and histidine, acting as nucleophile, electrophile and base respectively. The order of the amino acids varies between the clans (Rawlings and Barrett, 1994b). A number of mainly bacterial serine peptidases, however, utilise serine and lysine as their catalytic residues (clans SE and SF; Barrett and Rawlings, 1995). Eukaryotic serine peptidases comprise two main groups, regulatory

peptidases with high specificity and digestive enzymes with broader substrate use, such as trypsin and chymotrypsin.

Cysteine peptidases have also been known previously as thiol peptidases. Their activity depends on a catalytic dyad of cysteine and histidine residues (Rawlings and Barrett, 1994a). As with the catalytic triad of the serine peptidases, the order of the cysteine and histidine residues differs between the different families of cysteine peptidases. A wide variety of cysteine peptidases have been described, with the best known and largest family being the papain family (family C1, clan CA). These enzymes are generally lysosomal or secreted and include the major mammalian cysteine peptidases, cathepsins B, H, K, L and S (Rawlings and Barrett, 1994a). They have been shown to mediate extracellular matrix turnover, antigen presentation, and processing events. Furthermore, they might constitute potent drugs targets for osteoporosis, arthritis, immune-related diseases, atherosclerosis and cancer, as well as for numerous parasitic infections (Lecaille et al., 2002). Clan CD cysteine peptidases appear to be more specific in their function and include the caspases, peptidases involved in inflammatory disease and apoptosis (Mottram et al., 2003).

Metallo-peptidases are a vast group of enzymes that use a metal ion in the catalytic process, usually zinc, but also cobalt or manganese (Rawlings and Barrett, 1995a). Examples of eukaryotic metallo-peptidases are regulatory peptidases and matrix degradation enzymes, e.g. elastases and collagenases.

Aspartic peptidases need two aspartate residues for catalytic activity and are the simplest subclass of peptidases known. Family A1 of aspartic peptidases has one aspartate residue in each half of a bilobed protein, whilst family A2 consists of peptidases of a single lobe, again containing only one aspartate residue and which are therefore thought to be active as a homodimer (Rawlings and Barrett, 1995b). Aspartic peptidases are often secreted, with eukaryotic aspartic peptidases including digestive enzymes and intracellular lysosomal peptidases, though there are also a number of intramembrane peptidases (Rawlings and Barrett, 1995b).

Threonine peptidases were recognised more recently, with the discovery of the mechanism of action of the 26S proteasome (Seemuller et al., 1995). The active site threonine residue is at the amino terminal end of the peptidase and a second amino acid, lysine, is believed to be necessary for activity of the proteasome

(Seemuller et al., 1995; Bochtler et al., 1999). Non-proteasome threonine peptidases have also been identified (Bochtler et al., 1999).

Glutamic peptidases were identified in 2004 (Fujinaga et al., 2004), with only one family so far recognized. Their activity is not fully determined, but is likely to depend on a glutamine and glutamic acid catalytic dyad (http://merops.sanger.ac.uk/).

1.3.3 Peptidase inhibitors as drugs

Peptidase inhibitors can be classified as two types: low molecular weight peptidomimetic inhibitors and protein inhibitors. Inhibition can occur by two mechanisms: irreversible trapping reactions, which alter conformation and covalently bind the enzyme to prevent its participation in further reactions, and reversible reactions, where the inhibitor blocks the active site of the peptidase, either directly or indirectly (competitive or allosteric inhibition), but the inhibitor can dissociate from the enzyme (Fear et al., 2007). Peptidases are increasingly being seen as potential drug targets (Rawlings, 2007), and it has been estimated that 14% of human peptidases are being investigated by pharmaceutical companies (Southan, 2001). Therapeutically, peptidase inhibitors have been successfully introduced to treat HIV, hypertension, pancreatitis and multiple myeloma.

The HIV protease retropepsin cleaves some unique bonds, post phenylalanine residues, which has eased the task of designing selective inhibitors for the viral protease (Patick and Potts, 1998). There are eight inhibitors of the HIV protease that have been approved for use, with several more in clinical trials (Fear et al., 2007). They are commonly used in combination therapy with reverse transcriptase inhibitors, though they have also been found to be effective when used in monotherapy (Arribas et al., 2005). The introduction of protease inhibitors to combination therapy was associated with a substantial increase in the survival rates of people diagnosed with AIDS (Schwarcz et al., 2000).

Inhibitors of angiotensin-converting enzyme (ACE) inhibit the renin-angiotensin system and reduce blood pressure by decreasing peripheral vascular resistance. They have been used clinically since 1977 (Fear et al., 2007). ACE inhibitors have the advantage of better tolerability, limited side effects and a favorable metabolic profile compared to other antihypertensives (Ibrahim, 2006) and they also reduce

proteinuria and stabilise renal function, meaning they are also used for the treatment of diabetic nephropathy (Fear et al., 2007).

Acute pancreatitis is commonly treated with peptidase inhibitors, as these are considered to reduce damage to the pancreas, lessen systemic complications and reduce mortality. However, meta-analysis has shown that protease inhibitors only reduce mortality in patients with moderate to severe disease (Seta et al., 2004).

The proteasome inhibitor bortezomib is used to treat multiple myeloma and relapsed mantle cell lymphoma and is being investigated for other non-Hodgkin's lymphoma types. It causes cancerous cells to arrest in the G2/M phase of the cell cycle and to undergo apoptosis (Barr et al., 2007). Other proteasome inhibitors are also being investigated in clinical trials for multiple myeloma.

1.3.4 Peptidases as potential drug targets

As described previously, peptidases have been successfully exploited as drug targets and so other such enzymes are being investigated as therapeutic targets. Diseases for which peptidase inhibitors are being investigated include numerous infectious diseases, particularly viruses; various forms of cancer; diabetes mellitus; various inflammatory diseases and Alzheimer's disease (Rawlings, 2007).

Several inhibitors of viral peptidases are being tested in clinical trials, including two against the hepatitis C virus NS3/4A serine peptidase and two against picornavirus 3C serine peptidase (Fear et al., 2007). Additionally, the 3CL cysteine peptidase of severe acute respiratory virus (SARS) is known to be similar to the C3 peptidase of picornaviruses and *in vitro* the anti-picornavirus peptidase inhibitors were effective against SARS. SARS patients treated with the HIV protease inhibitors lopinavir and ritonavir in combination have also been shown to have better clinical outcomes (Fear et al., 2007). Inhibitors of the peptidases of cytomegaloviruses, herpes simplex viruses and rotaviruses are also being developed.

Serine, cysteine and metallo-peptidases are implicated in cancer metastasis and so inhibitors of these enzymes are being investigated. Inhibitors of specific zinc matrix metalloproteases (MMP) have been researched as anti-cancer agents since the 1980s, but have so far shown disappointing efficacy, with significant side

effects and a lack of specificity (Fingleton, 2006). Despite the failure of many clinical trials, phase III trials of more specific MMP inhibitors are continuing for pancreatic, non-small-cell lung and renal cell carcinomas (Coussens et al., 2002). Inhibitors of other peptidases are also being investigated as anti-cancer agents, with inhibitors of the serine peptidase urokinase plasminogen-activating enzyme being evaluated in phase I clinical trials and a wide variety of inhibitors of cathepsin B, which is over-expressed in cancer cells, are being researched, though none have yet reached clinical trial (Fear et al., 2007).

Inhibitors of the serine peptidase dipeptidyl peptidase IV are proposed for treating diabetes mellitus. This peptidase degrades peptides involved in stimulating pancreatic beta cells to produce insulin and several inhibitors are in clinical trial to assess their enhancement of the insulin response (McIntosh et al., 2006).

In inflammatory diseases, peptidase inhibitors have been investigated for use as anticoagulants, by binding to the serine peptidase thrombin; to minimise reperfusion injury; to reduce cerebral inflammation due to MMP-2 and -9 after stroke injury; for the reduction of atherosclerosis caused by MMP, cathepsins S, K, L and neutrophil elastase; to inhibit pulmonary inflammation, caused by elastase, collagenase and cathepsin G; and to reduce gastrointestinal inflammation by inhibiting trypsin (Fear et al., 2007).

The aspartic peptidases β- and γ-secretase are involved in the formation of the plaques found on the brain in Alzheimer's disease and inhibition of these peptidases is being explored as a new treatment option. Inhibitors of β-secretase are yet to enter clinical trial, whilst inhibitors of γ-secretase have been found to be well tolerated in phase I trials (Siemers et al., 2005) and are being evaluated in phase II trial (Fear et al., 2007).

The peptidases of various protozoan parasites are thought to be promising drug targets. Cysteine and serine peptidases of *Plasmodium* have been found to mediate invasion of erythrocytes by merozoites and the rupture of erythrocytes by schizonts, with inhibitors of both blocking these processes. Inhibitors of cysteine peptidases and aspartic peptidases have also been found to block *P. falciparum* development. Peptide-based inhibitors of falcipain, a cathepsin L-like cysteine peptidase found in the food vacuole of malaria, have been shown to cure 80% of murine malaria (Rosenthal, 1999). The proteasome of *P. falciparum* is also a

promising drug target, with the multiple myeloma drug bortezomib being a potent inhibitor of the erythrocytic stage of both drug-sensitive and resistant *P. falciparum* strains, inhibiting parasite development and multiplication (Reynolds et al., 2007).

Peptidyl proteasome inhibitors have also been found to be effective anti-African trypanosomal agents, suggesting inhibition of the proteasome would be a good target for treatment of *T. brucei* (Nkemgu et al., 2002). The *T. brucei* lysosomal cathepsin L-like cysteine peptidase trypanopain is also a potential drug target, with several inhibitors of trypanopain killing cultured bloodstream form *T. brucei* and the most effective inhibitors protecting mice from an otherwise lethal infection in an *in vivo* model of acute infection (Troeberg et al., 2000). For *T. cruzi* the lysosomal cysteine peptidase cruzipain is being investigated as a therapeutic target, since cysteine peptidase inhibitors which target cruzipain have shown ability to rescue mice from acute lethal infections of *T. cruzi* and have cured mice in the chronic stage of disease (Engel et al., 1998). An inhibitor of cruzipain is in the early stages of clinical trials (Jaishankar et al., 2008).

1.3.5 Peptidases in *Leishmania*

A total of 154 peptidases were found to be present in the *L. major* genome (Ivens et al., 2005), with examples of serine, cysteine, aspartic, threonine and metallo-peptidases, as well as one peptidase of unknown catalytic type. The peptidases make up around 1.8% of the genome and are illustrated in Figure 1.6.

1.3.5.1 Aspartic peptidases

Two aspartic peptidases were found in the *L. major* genome sequence (Ivens et al., 2005), one with sequence similarity to presenilin 1 (PS1), which is a multi-pass membrane peptidase, and the other which has sequence similarity to an intramembrane signal peptide peptidase (SPP). PS1 cleaves type I membrane proteins, which are membrane proteins which have a single transmembrane domain, with the carboxyl end of the protein in the cytoplasm. In *L. major* PS1 is potentially involved in autophagy. SPP cleaves the transmembrane domains of signal peptidases (Besteiro et al., 2007).

However, a soluble peptidase inhibited by aspartic peptidase inhibitors and which could cleave a substrate specific for the aspartic peptidase cathepsin D, has been

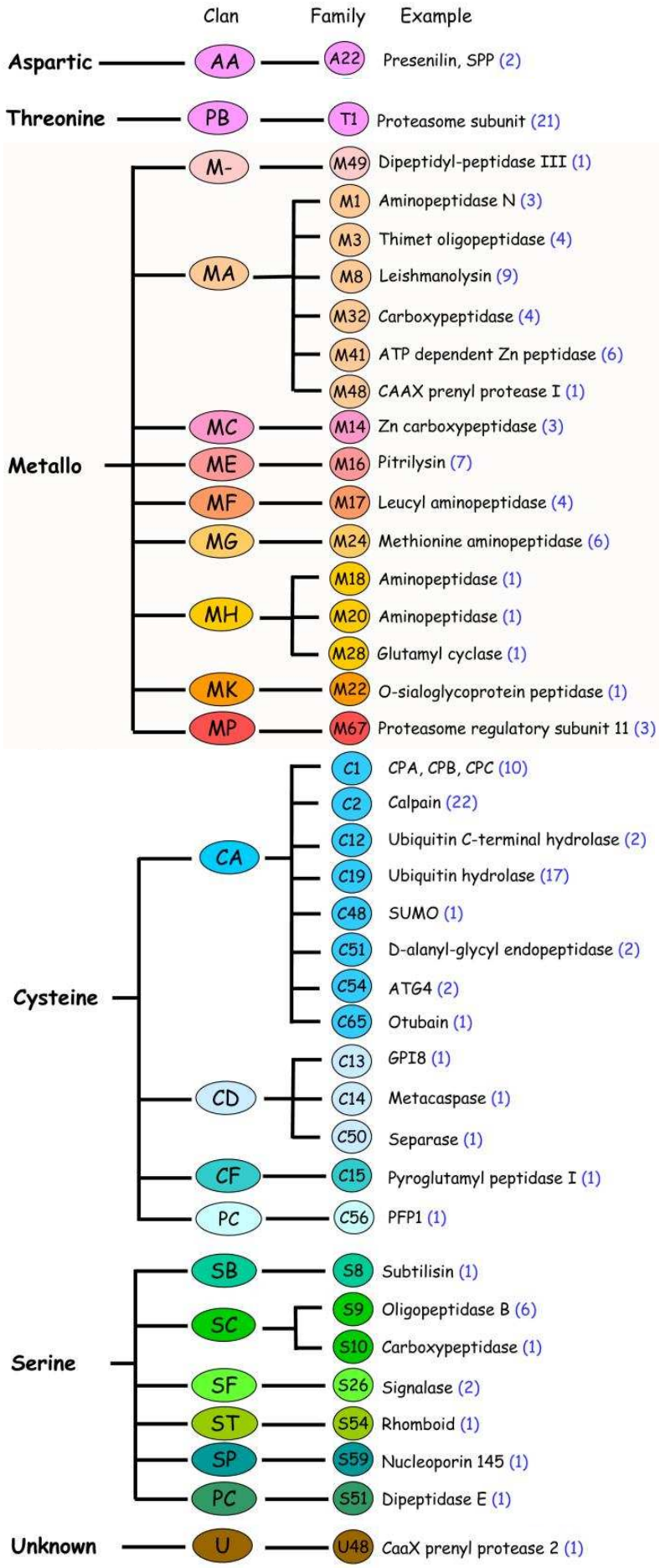


Figure 1.6 Clans and families of *L. major* peptidases. Nomenclature is based on the MEROPS database (http://merops.sanger.ac.uk/). Numbers in brackets represents the estimated number of peptidases in each family. Figure taken from Besteiro et al., 2007.

reported in *L. mexicana* (Valdivieso et al., 2007). An aspartic peptidase inhibited by pepstatin A has also been demonstrated to be present in soluble extracts of *L. amazonensis*, being found to be at its highest level in promastigotes and in the early stages of differentiation to amastigotes *in vitro* (Alves et al., 2005). These results suggest that either one of the peptidases found in *L. major* is actually a soluble peptidase or there may be species-specific differences in the peptidases present.

1.3.5.2 Threonine peptidases

Twenty-one threonine peptidases were found in the *L. major* genome, though all are classified as proteasome subunits (Besteiro et al., 2007). The proteasome is a multisubunit, multicatalytic peptidase responsible for degradation of ubiquitinated proteins in the cytosol (Silva-Jardim et al., 2004). Sequences of both types of threonine proteasomes were found in *L. major*, i.e. the eukaryotic-type 20S and the bacterial-type *hsl*V proteasomes (Gille et al., 2003). The finding of numerous proteasome subunits in *L. major* is to be expected, as at least 10 subunits of varying size were found in *L. mexicana* (Robertson, 1999) and at least 6 subunits were found in *L. chagasi* (Silva-Jardim et al., 2004), based on electrophoresis of purified proteasomes.

As with the malarial and *T. brucei* proteasomes, the proteasome of *Leishmania* is a potential therapeutic target, as the use of specific inhibitors has shown the proteasome to be necessary for growth of *L. mexicana* promastigotes and amastigotes *in vitro* (Robertson, 1999). Proteasome inhibitors also inhibited the growth of *L. chagasi* promastigotes and reduced amastigote survival within macrophages (Silva-Jardim et al., 2004). In addition, two HIV protease inhibitors, which inhibit the proteasome, have been shown to be leishmanicidal against *L. major* and *L. infantum* promastigotes (Savoia et al., 2005).

1.3.5.3 Metallo-peptidases

16 families of metallo-peptidases were identified in *L. major* (Besteiro et al., 2007), though only those of clan MA, family M8 (the leishmanolysin or GP63 family), have been comprehensively studied. GP63 is the major glycosyl phosphatidyl inositol (GPI)-anchored surface protein of *Leishmania* promastigotes and aids the uptake of *Leishmania* by macrophages. GP63 binds complement component C3 and

converts this to its breakdown products, which interact with macrophage complement receptors, facilitating the uptake of the promastigotes by macrophages. GP63 is also thought to prevent lysis of the parasites by binding and breaking down C3 and to protect amastigotes from degradation in the parasitophorus vacuole of macrophages (Yao et al., 2003), and has also been found to protect *L. amazonensis* and *L. major* from various antimicrobial peptides (Kulkarni et al., 2006).

Four members of the leucyl aminopeptidase family (clan MF, family M17) were found in the *L. major* genome (Ivens et al., 2005). One of these, leucyl aminopeptidase (Lap), has been investigated in *L. major*, *L. amazonensis* and *L. donovani* (Morty and Morehead, 2002). The substrate specificity of recombinant Lap was determined, with zinc found to be the likely natural cofactor and L-Leu-AMC (7-amino-4-methylcoumarin) the best substrate for the Lap enzymes of all three *Leishmania* species. Both metal ion chelators and anti-Lap IgG inhibited the activity of recombinant Lap and by using anti-Lap IgG, Lap was found to be responsible for the majority of the L-Leu-AMC hydrolysis in soluble parasite extracts, though other, unidentified, aminopeptidases in addition to Lap were responsible for L-Cys-AMC and L-Met-AMC hydrolytic activity (Morty and Morehead, 2002). Malaria Lap has shown promise as a drug target (Nankya-Kitaka et al., 1998; Howarth and Lloyd, 2000), but this has not been investigated for *Leishmania*.

There were six methionine aminopeptidases found in the *L. major* genome (clan MG, family M24), and two specific methionine aminopeptidase inhibitors have previously been found to inhibit the growth of *L. donovani* to a similar extent as the antileishmanial drug pentamidine (Zhang et al., 2002).

Aside from these examples, the specific metallo-peptidases of *Leishmania* have not been investigated, though unidentified metallo-peptidases inhibited by 1,10 phenanthroline were found in soluble extracts of *L. amazonensis*. This peptidase or peptidases had higher activity in promastigotes and in the early hours of differentiation to amastigotes *in vitro* than in amastigotes (Alves et al., 2005).

1.3.5.4 Cysteine peptidases

L. major was found to have members of four clans of cysteine peptidases, with enzymes of eight families of clan CA, three families of clan CD and one family each of clans CF and PC (Ivens et al., 2005).

The peptidases of the papain family (clan CA, family C1) are the most extensively studied peptidases of *Leishmania*, comprising the cathepsin-L like peptidases CPA and CPB, and the cathepsin-B like peptidase CPC, all of which are lysosomal. There is one copy of *CPA* and *CPC* in *Leishmania*, whilst *CPB* is found in a tandem array of genes, with eight genes in *L. major* and 19 in *L. mexicana*, the species primarily used for characterisation of the peptidase. Both the copy number of *CPB* genes and the sequences of the genes vary between the different *Leishmania* species (Hide et al., 2007). Neither CPA nor CPC are essential for survival of *L. mexicana* in the host, with no defect in the ability of *CPA* null mutants to infect mice (Souza et al., 1994), and whilst *CPC* null mutants had a reduced infectivity to macrophages, there was no defect in the growth of lesions in mice (Bart et al., 1997). However, CPB has been found to be a virulence factor, with deletion of the *CPB* array leading to slower appearance of small lesions in mice (Mottram et al., 1996b). In addition, double null mutants for *CPB* and *CPA* did not produce lesions in mice, suggesting exacerbation of the phenotype (Alexander et al., 1998). The *CPA/CPB* double null mutants have a defect in autophagy, which is thought to lead to their defect in differentiation to metacyclics and amastigotes (Williams et al., 2006).

The use of an inhibitor specific to cathepsin L-like cysteine peptidases, K11777, has shown that these peptidases are necessary for the growth of *L. tropica* and *L. major* promastigotes and for the growth of lesions of *L. tropica* in mice, indicating the potential of the peptidases as a drug target (Mahmoudzadeh-Niknam and McKerrow, 2004). Inhibitors of all three peptidases have also been tested and found to inhibit promastigote growth, infection of macrophages and development of lesions in mice, being selective for parasite cysteine peptidases over mammalian peptidases (Selzer et al., 1999). These peptidases have also been suggested to have a role as virulence factors by modulating the immune response. Splenocytes from mice infected with *L. mexicana CPA/CPB* double null mutants increased their production of Th1-type response cytokines, and reduced production of IL-4, a Th2-type response cytokine (Alexander et al., 1998) and mice

infected with *L. mexicana CPB* null mutant promastigotes controlled the infection with a Th1-type response (Buxbaum et al., 2003), with multiple *CPB* isoforms being necessary to restore a Th2-type response (Denise et al., 2003). CPC has also been shown to have a role in the exacerbation of disease, as *L. chagasi* CPC cleaved the cytokine TGF-β from its precursor form to the active form, which aids survival in macrophages and inhibits Th1-type cytokines (Somanna et al., 2002). In addition, CPA and CPB have been shown to be antigens in human disease, as sera from leishmaniasis patients reacts with recombinant *L. major* CPA and CPB (Rafati et al., 2001).

In addition to these well characterised papain family enzymes, *L. major* contain many other clan CA peptidases. There are 22 members of the calpain family, which in higher eukaryotes are central in calcium-regulated functions including signal transduction, cell differentiation and in apoptosis. Their role in *L. major* has yet to be elucidated (Besteiro et al., 2007). *L. major* also has numerous enzymes involved in ubiquitination; with 19 ubiquitin hydrolases (families C12 and C19), one small ubiquitin-like modifier (SUMO)-specific peptidase (family C48) and one otubain (family C65). These ubiquitination-associated genes work in partnership with the proteasome (discussed in Section 1.3.2.2), and the number of genes present indicates a nonlysosomal cytosolic protein degradation system is important (Ivens et al., 2005). Two members of the C51 family of d-alanyl-glycyl endopeptidases or peptidoglycan amidases are present in *L. major*, which contain CHAP (cysteine, histidine-dependent amidohydrolases/peptidases) domains. In bacteria, these enzymes are involved in cell division, growth and cell lysis (Bateman and Rawlings, 2003), but are as yet uninvestigated in *Leishmania*. There are also two members of the C54 family, the ATG4 peptidases, which have a key role in the autophagic pathway, with the ATG4 peptidase processing ATG8 and being crucial for autophagosome function. Null mutants for one of the isoforms, *ATG4.2*, have been produced. They have a defect in both autophagy and in differentiation to metacyclic promastigotes, again suggesting autophagy is necessary for this differentiation (Besteiro et al., 2006).

Members of three clan families of CD cysteine peptidases were found in *L. major*: GPI8 from family C13, metacaspase from family C14 and separase from family C50. GPI8 is the catalytic component of the GPI:protein transamidase complex which adds GPI anchors to prescursor GPI-anchored proteins in the endoplasmic reticulum. The gene has been shown to be non-essential in *L. mexicana*, with null

mutants being able to differentiate into amastigotes and establish infections in purified macrophages and mice (Hilley et al., 2000). Metacaspases are evolutionary distant orthologues of metazoan caspases which have been identified only in plants, fungi, protozoa and some bacteria (Gonzalez et al., 2007). Metacaspases are thought to have a key role in inducing programmed cell death (PCD) in plants and yeast and the *L. major* metacaspase has been shown to complement a PCD phenotype in yeast (Gonzalez et al., 2007). Two metacaspase genes have been identified in *L. donovani*, which were localised to acidocalcisomes and found to have a trypsin-like specificity for cleavage of substrates. The *L. donovani* metacaspases are suspected to have a role in PCD, as they were upregulated in parasites undergoing PCD and promastigotes over-expressing metacaspase 1 were more sensitive to hydrogen peroxide-induced PCD (Lee et al., 2007). In *L. major* there is only one metacaspase gene, which was located in punctate structures in the cytoplasm in interphase cells, but was concentrated in the kinetoplast when this was segregating and was localised in the nucleus during mitosis. It was not possible to generate metacaspase null mutants, suggesting it is essential for the correct segregation of the nucleus and kinetoplast, and it is a potential drug target, particularly with the absence of metacaspase from mammals (Ambit et al., 2008). In higher eukaryotes separase is a major regulator of the metaphase-to-anaphase transition. Little is known about chromosome segregation in *Leishmania*, but separase is likely to have a crucial role in the parasite's cell cycle control mechanisms (Besteiro et al., 2007).

The clan CF, family C15 cysteine peptidase that was found in *L. major* was pyroglutamyl peptidase I (PPI), which removes N-terminal l-pyroglutamyl residues (l-pGlu), post-transcriptional modifications conferring relative aminopeptidase resistance that are essential to the modified peptides' biological activity in some cases. The *L. major* PPI has been previously cloned and expressed as a recombinant enzyme, which had pyroglutamyl peptidase activity. It was possible to generate *LmjPPI* null mutants, which were able to differentiate to metacyclic promastigotes and successfully infect murine macrophages. It is thought that PPI could regulate l-pGlu-modified peptides which are necessary for differentiation of metacyclic promastigotes since over-expression of PPI caused a defect in metacyclogenesis (Schaeffer et al., 2006).

Only one member of the family C56, clan PC was found in *L. major*, *Pyrococcus furiosus* protease 1 (PFP1). This is a multimeric cysteine peptidase, which forms

a doughnut-shaped hexamer, made up of a trimer of dimers. This peptidase has limited taxonomic distribution, with homologues being found in certain bacteria, archaea and plants. A PFP1-like protein is expressed in *L. major*, but not in other species of *Leishmania* or trypanosomes. Pseudogenes of PFP1 have been found in *L. mexicana*, *L. braziliensis* and *L. infantum*. The expression of *PFP1* in *L. major* suggests it may contribute to the disease tropism that distinguishes *L. major* from other *Leishmania* species, or it may be becoming a pseudogene, albeit more slowly than in other species (Eschenlauer et al., 2006).

1.3.5.5 Serine peptidases

The trypsin/chymotrypsin family (S1) is one of the most abundant groups of serine peptidases, but none were identified in *L. major* (Ivens et al., 2005). Seven other serine peptidase families were identified. There is one subtilisin-like serine peptidase, which has a signal peptide and is likely to be part of the secretory / endosomal system, involved in the processing of secreted proteins. The subtilisin-like peptidases of *Plasmodium* are thought to be potential drug targets (Withers-Martinez et al., 2004). The other poorly characterised serine peptidases are: one lysosomal serine carboxypeptidase, a homologue of which has previously been characterised in *T. cruzi* (Parussini et al., 2003); two members of the signalase family, type-I signal peptide peptidases, which cleave signal peptides from proteins in the endoplasmic reticulum (Tuteja, 2005); a 26S regulatory subunit of the proteasome; a nucleoporin and a rhomboid-like protein (Ivens et al., 2005).

There are also six members of the prolyloligopeptidase family (S9), including prolyloligopeptidase (POP), peptidyl-dipeptidase IV and oligopeptidase B (OPB). OPB has been shown to be a virulence factor in *T. cruzi*, mediating entry into host cells (Caler et al., 1998) and the OPB of African trypanosomes is released into the host serum, where it cleaves host blood factors (Morty et al., 2005a). *T. cruzi* POP is also involved in entry of parasites to host cells, with inhibitors preventing host cell invasion (Grellier et al., 2001). A likely *Leishmania* OPB was described in *L. amazonensis* in 1998 (de Andrade et al., 1998) and subsequently in *L. major* in 1999 (Morty et al., 1999a). The *L. amazonensis* OPB was later cloned, sequenced and found to be 90% identical to *L. major* and *L. infantum* OPB and 84% identical to *L. braziliensis* (de Matos Guedes et al., 2007). Whether OPB has a virulence role in *Leishmania*, as in trypansomes, was unknown; addressing this question comprised part of my project.

As well as these defined serine peptidases, three other unidentified serine peptidase activities have been investigated in *L. amazonensis*. Firstly, a 68 kDa soluble serine peptidase was found which could cleave haemoglobin, bovine serum albumin (BSA), ovalbumin, arginine-containing peptide substrates and insulin, being found to prefer to cleave insulin after hydrophobic residues or glutamate residues (da Silva-Lopez and Giovanni-De-Simone, 2004). The activity was inhibited by *N*-tosyl-L-phenylalanyl-chloromethylketone (TPCK) and antipain, inhibitors of chymotrypsin-like and trypsin-like peptidases respectively. This peptidase was subsequently localised to vesicular structures close to the flagellar pocket, morphologically similar to those of the endocytic/exocytic pathway (Morgado-Diaz et al., 2005).

Secondly, a secreted serine peptidase has been characterised. This 56 kDa peptidase appears to be present as a homodimer and could also cleave a number of substrates, including haemoglobin, BSA, ovalbumin, fibrinogen, collagen type II and arginine-containing peptide substrates, again cleaving substrates after hydrophobic residues (Silva-Lopez et al., 2005). This activity was only fully inhibited by aprotinin, but also partially by benzamidine, TPCK and phenylmethylsulfonyl fluoride (PMSF); aprotinin and benzamidine are inhibitors of trypsin-like peptidases, whilst TPCK is an inhibitor of chymotrypsin-like inhibitors. This peptidase was found to be secreted through the flagellar pocket, being predominantly located in the flagellar pocket and in endocytic/exocytic vesicles in promastigotes, and in megasomes as well as the flagellar pocket in amastigotes (Silva-Lopez et al., 2004).

Finally, an unidentified serine peptidase activity, inhibited by PMSF, has also been found in soluble extracts of *L. amazonensis*, with higher activity in promastigotes and in the early hours of differentiation to amastigotes *in vitro* than in amastigotes (Alves et al., 2005).

Investigation of the effect of serine peptidase inhibitors on the survival of *Leishmania* has shown that TPCK and benzamidine both reduce viability and induce morphological changes in the *L. amazonensis* promastigotes (Silva-Lopez et al., 2007), suggesting serine peptidases could be useful potential drug targets.

1.4 Aims of Project

The peptidases of *Leishmania* described above, particularly those identified by the genome but so far not explored, have been deemed to be a fruitful line of research for in depth characterisation and investigation of their potential use as drug targets. Thus, the research described in this thesis had the following four aims:

1) to develop an assay to characterise peptidase activity in live *Leishmania* promastigotes and to identify the major peptidase(s) involved,
2) to fully characterise the identified peptidase(s),
3) to evaluate its (their) potential as an antileishmanial target using specific inhibitors and genetic manipulation, and
4) to determine the value of the luciferase reporter gene for identifying the presence of *Leishmania* in infected macrophages.

Chapter 2

MATERIALS & METHODS

2.1 Cell Culture

2.1.1 *Leishmania* spp promastigote culture

Leishmania promastigote parasites (*L. mexicana* MNYC/BZ/M379*, L. major* MHOM/IL/80/Friedlin and *L. infantum* JPC clone M5, MCAN/ES/98/LLM-877), were maintained in culture in HOMEM medium (Invitrogen, Paisley, UK, ref 041-946-99M), with 10% (v/v) heat-inactivated foetal calf serum (HIFCS; Labtech International, Ringmer, UK). Starting inoculation was ~10^5 cells/ml and cells were sub-passaged once grown to stationary phase, ~3 x 10^7 cells/ml, after around 1 week. Cultures were grown at either 25℃ (*L. mexicana* and *L. major*) or 27℃ (*L. infantum*), with air as the gas phase, in phenolic-style lidded flasks.

To start initial cultures, amastigotes were isolated from either a footpad lesion on a BALB/c mouse (*L. mexicana* and *L. major*) or from a Golden hamster spleen (*L. infantum*), by either Mrs Susan Baillie or Mrs Denise Candlish (both University of Glasgow), and differentiated by inoculation into HOMEM with 10% (v/v) HIFCS into promastigotes. *Leishmania* were used in experiments up to sub-passage 20.

When necessary, antibiotics were added to the cultures of generated transgenic cell lines as follows: hygromycin B at 50 µg/ml, neomycin (G418, Geneticin) at 12.5 to 50 µg/ml (both Calbiochem, Nottingham, UK) and nourseothricin at 75 µg/ml (Hans Knoll Institute, Jena, Germany).

Stabilates of *Leishmania* were made by diluting 500 µl of mid log phase cells with 500 µl of a mixture of 30% glycerol / 70% HIFCS in a 1.5 ml cryotube, slow cooling these stabilates using an isopropanol bath to –80℃ , storing them overnight at –80℃ and then indefinitely in liquid nitrogen.

2.1.2 Growth of *Leishmania* spp axenic amastigotes

L. mexicana promastigotes were transformed to axenic amastigotes by a 1 in 10 dilution of stationary phase promastigotes into Schneider's Drosophila medium containing 10% (v/v) HIFCS, 0.3% (v/v) Hemin (from a 2.5 mg/ml stock in 50 mM NaOH), at pH 5.5 and in a 32.5℃ incubator with 5% CO_2. They were maintained by weekly subpassage, of 1 in 10 dilution in the same medium, in vented lidded flasks. To transform parasites back into the promastigote stage, 1 ml of axenic

amastigotes was transferred into 9 ml of HOMEM medium, with 10% (v/v) HIFCS and incubated at 25℃ with air as the gas phase.

2.1.3 Growth of THP-1 cells

The human monocytic cell line THP-1 was originally cultured from the blood of a boy with acute monocytic leukaemia (Tsuchiya et al., 1980). THP-1 cells (a gift of Dr Vanessa Yardley, LSHTM, UK), were maintained in RPMI 1640 medium (PAA Cell Culture, Yeovil, UK), with 10% (v/v) HIFCS, 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, Poole, UK), and incubated at 37℃ with 5% CO_2. They were sub-passaged twice-weekly, splitting the flask 1 in 4 with medium, with a starting inoculum of ~10^5 cells/ml in vented lidded flasks.

2.1.4 Isolation of *L. major* metacyclic promastigotes

Isolation of *L. major* metacyclic promastigotes was completed based on the method of Sacks (Sacks et al., 1985). *L. major* promastigotes were counted, then centrifuged for 10 minutes at 1000 *g* at room temperature. They were washed in PBS, recentrifuged and resuspended at 2 x 10^8 parasites/ml in PBS. Sterile peanut agglutinin was added to the cell suspension at a final concentration of 100 µg/ml. The tube was mixed and incubated stationary for 30 min at room temperature. The supernatant was carefully removed from the pellet and the number of metacyclic promastigotes in this supernatant were counted. The metacyclic promastigotes were then adjusted to the desired concentration for use. In order to calculate the percentage of metacyclic promastigotes in a culture of stationary phase cells, the number of metacyclic promastigotes found after the incubation with peanut agglutinin was compared to the initial concentration of 2 x 10^8 parasites/ml. Two replicates were completed per parasite line.

2.1.5 Determination of cell densities

2.1.5.1 Promastigote parasites

An equal volume of culture and 1% formaldehyde in PBS were combined and 10 µl of the fixed cells was pipetted onto a Neubauer haemocytometer and left to settle to the bottom at room temperature. Five of the large squares were counted, and the count multiplied by 10^5 to give the number of cells present in 1 ml of culture.

2.1.5.2 Axenic amastigote parasites

Amastigotes were separated by passage through a 26 gauge needle. 10 µl of cells were then pipetted onto a Neubauer haemocytometer and left to settle to the bottom at room temperature. 5 of the large squares were counted, and the count multiplied by 5 x 10^4 to give the number of cells present in 1 ml of culture.

2.1.5.3 THP-1 cells

10 µl of THP-1 cells were pipetted onto a Neubauer haemocytometer and left to settle to the bottom at room temperature. Five of the large squares were counted, and the count multiplied by 5 x 10^4 to give the number of cells in 1 ml of culture.

2.1.6 Harvest, lysis and fractionation of cells

Parasites or macrophage cells were harvested by centrifugation at 1300 *g* at room temperature for 10 min, washed twice in PBS and either used immediately or stored at -80℃ until used. Two methods were used to lyse cells. Cells were either lysed by resuspension to the required concentration of cells per ml in lysis buffer (50 mM Tris/HCl pH 8.0, 0.25% (v/v) Triton X-100, 20% (v/v) glycerol), or by sonication of cells suspended in PBS, using a Soniprep 150 (MSE Ltd, Lower Sydenham, UK) on full power 3 times for 30 sec with 10 sec in between. As necessary, the following peptidase inhibitors were included, either singly or in combination, to prevent peptidase activity in the lysates: 10 µM E-64 (*trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane), 400 µM 1,10-phenanthroline, 2 µM pepstatin A, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 µM antipain, 10 µM leupeptin and 2 mM ethylenediamine tetra acetic acid (EDTA).

To separate lysates into membrane and soluble fractions, the lysate was spun at 37500 *g* for 1 h at 4℃. The supernatant (soluble fraction) was separated from the pellet (membrane fraction), and the pellet was washed with PBS, recentrifuged and then resuspended in the same volume as before in lysis buffer.

2.1.7 Differentiation of THP-1 cells

THP-1 cells can be differentiated from a suspension of monocytic cells to attached macrophage like cells by the addition of phorbol esters (Tsuchiya et al., 1982;

Wang et al., 1996). Based on these methods and that of Ogunkolade et al. for the infection of THP-1 cells with *Leishmania* (Ogunkolade et al., 1990), 20 nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, Poole, UK), from a stock of 100 µM was added to THP-1 cells diluted to a concentration of 5 x 10^5 cells/ml. Cells were added to the wells of a 12 well plate and left to adhere for 2 or 3 days at 37℃ with 5% CO_2. THP-1 cells were then treated as mouse macrophages for infection with *Leishmania* promastigotes or immunofluorescence (Sections 2.2.2 and 2.6.5.2).

2.2 Infectivity of *Leishmania* spp

2.2.1 Harvest of peritoneal exudate macrophages (PEM)

Female CD1 mice were culled, the abdomen was degloved and 10 ml of RPMI 1640 medium containing 1% (v/v) gentamicin and 1% (v/v) penicillin/streptomycin was injected into the abdomen under the sternum using a 21 gauge needle. The mice were shaken to detach the macrophages into the medium. A 26 gauge needle was then used to extract the medium from the side of the abdomen. The PEM were centrifuged at 1000 *g* for 10 min at 4℃, then resuspended in fresh RPMI 1640 medium with 10% (v/v) HIFCS and counted. The PEM were adjusted to 5 x 10^5 cells/ml in the same medium, 100 µl of this was added to each well of Nunc 16-well Lab-tek™ tissue culture slides (Fisher Scientific, Loughborough, UK) and these were incubated at 37℃ with 5% CO_2 overnight. PEM were generally infected with *Leishmania* the day following extraction, but they could be infected up to one week after extraction.

2.2.2 Infectivity *in vitro* in PEM

PEM were infected with cultured promastigotes or axenic amastigotes. Stationary phase promastigotes, metacylic promastigotes or axenic amastigotes were counted as detailed above (Sections 2.1.5.1 and 2.5.1.2) and diluted in RPMI 1640 with 10% (v/v) HIFCS to the relevant concentration for the desired ratio. 100 µl was added to the 16-well chamber slides already containing PEM. Slides were incubated at 32.5℃ (*L. mexicana)*, or 37℃ (*L. major* or *L. infantum*) with 5% CO_2. Parasites were left to infect overnight then the slides were washed at least twice with RPMI 1640 and the macrophages were re-covered with 200 µl RPMI 1640 with 10% (v/v) HIFCS.

The slides could be stopped at this 24 h point, or kept for up to 1 week at the relevant temperature to determine how the infections changed over time. Slides left to incubate for over four days had their medium replaced at this point.

When stopped, slides were fixed with 100% methanol for around 1 min and stained with 10% Giemsa's stain (VWR International, Lutterworth, UK) for 10 min. The percentage of macrophages infected and, if necessary, the number of amastigotes per macrophage were determined by light microscopy.

2.2.3 Assessment of infectivity in BALB/c mice

Groups of 5 or 6 female BALB/c mice were inoculated in the footpad with 5 x 10^5 stationary-phase *L. major* promastigotes resuspended in 20 µl of PBS, pH 7.4. The thickness of the footpad was measured weekly until the footpad was 5 mm thick, at which stage the mice were culled.

2.3 Testing the Effectiveness of Anti-Leishmanial Drugs

2.3.1 Alamar blue tests with promastigote cells

L. major cells were diluted to a concentration of 2 x 10^6 cells/ml in HOMEM medium with 10% (v/v) HIFCS for use.

Drugs were initially tested from a starting concentration of 10 µM, with an appropriate serial dilution. Drugs were made up in HOMEM medium with 10% (v/v) HIFCS, at twice the desired final concentration.

Tests were completed in 96 well plates, with 100 µl of the appropriate drug concentration added to the wells, and then 100 µl *Leishmania* was added to the drugged wells and to control wells. Control wells contained either 100 µl of HOMEM with 10% (v/v) HIFCS, for water soluble drugs, or 100 µl of a matched serial dilution of the liquid in which the drugs were made up in, for example DMSO or ethanol, in HOMEM with 10% (v/v) HIFCS.

Plates were incubated for 5 days at 25℃, with air as the gas phase. After 5 days, 20 µl sterile resazurin solution (0.0125% (w/v) resazurin salt (Sigma, Poole, UK) in PBS, filter sterilised), was added and the plates incubated for a further 24 h.

Plates were read using an EnVision plate reader (Perkin Elmer, Beaconfield, UK) at emission wavelength of 535 nm, excitation of 620 nm, with a general mirror. LD_{50} values were determined by comparison to the control wells, using GraFit 5 data analysis software (Erithacus Software, Horley, UK).

2.3.2 Testing of amastigotes within PEM

PEM were harvested and infected with promastigote *L. major* as described in Sections 2.2.1 and 2.2.2. PEM were infected at a 7:1 ratio of promastigotes:PEM. The un-engulfed promastigotes were washed off at the 24 h time point, and the drugs were added. Drugs were initially tested from a starting concentration of 10 µM, with a 3-fold serial dilution. They were made up in RPMI 1640 medium with 10% (v/v) HIFCS.

200 µl of the appropriate drug concentration was added to wells of the chamber slides, in triplicate or quadruplicate, with 200 µl of control solution added to control wells. This control solution was either RPMI 1640 with 10% (v/v) HIFCS for water soluble drugs, or a matched serial dilution of the solvent in which the drug was made up, in RPMI 1640 with 10% (v/v) HIFCS.

The slides were incubated at 37℃ with 5% CO_2 for 5 days, with the drugs completely replenished on the 3rd day. After the incubation, slides were fixed with 100% methanol for around 1 min and stained with 10% Giemsa's stain for 10 min. The percentage of macrophages infected was determined by light microscopy and IC_{50} values were determined by comparison to the control wells, using GraFit 5 data analysis software (Erithacus Software, Horley, UK).

2.4 Uptake and Cleavage of Fluorescent Molecules Observed with Fluorescent Plate Reader

2.4.1 Basic peptide-AMC cleavage assay

Either benzoyl-Arg-AMC (Bz-R-AMC), 3-carboxy-propionyl-Ala-Ala-Pro-Val-AMC (Suc-AAPV-AMC) or 3-carboxy-propionyl-Ala-Ala-Pro-Phe-AMC (Suc-AAPF-AMC; all Bachem, Weil am Rhein, Germany) were used to assess the enzymatic capacity of various *L. major* and *L. mexicana* WT and mutant promastigotes, both in log

phase (day 3 following sub-passage) and in stationary phase (day 7 following sub-passage). All were made up at a stock concentration of 100 mM as per the manufacturer's instructions.

Leishmania promastigotes were counted and diluted to the desired concentration (typically 6 x 10^6 cells/ml) in HOMEM medium. 100 µl of cell suspension was used per well in a black 96-well plate (Greiner Bio-one, Stonehouse, UK). Control wells of the same volume of HOMEM medium were included with every assay, to account for the inherent cleavage of the substrate.

The relevant peptide-AMC was diluted to 10 µM and 100 µl of this was added to both parasite and control wells to give a final concentration of 5 µM.

For each assay, an AMC standard was included. AMC (Calbiochem, Nottingham, UK) was made up as a stock of 1 µM and a two-fold serial dilution in water was included on each plate, starting from 50 nM. The values for the standard were converted into a standard curve to assess the fluorescence associated with a particular concentration of AMC.

Fluorescence was read every min for 30 repeats, in an EnVision™ Multilabel Reader (Perkin Elmer, Beaconfield, UK) at emission wavelength of 355 nm, excitation of 460 nm and using the general mirror. The fluorescence readings (minus the fluorescence of the control wells) were converted to a concentration of AMC released using the AMC standard. The figures were graphed using Microsoft Excel to determine the rate of cleavage of Bz-R-AMC over the 30 min time frame.

2.4.2 Inhibition of cleavage

To determine the effect of peptidase inhibitors or other compounds, the basic Bz-R-AMC assay was completed as described in Section 2.4.1, with minor changes. The inhibitor under test was made up at 20x the desired concentration. 10 µl of inhibitor was added to the desired wells of a black 96 well plate. *Leishmania* promastigotes were diluted to a concentration of 6.67 x 10^6/ml and 90 µl were added to the relevant wells. The HOMEM control and AMC standard were added to the plate as described in Section 2.4.1. 100 µl of 10 µM Bz-R-AMC was also added to the relevant wells to give a final concentration of 5 µM.

The following inhibitors and drugs were used, with these starting concentrations: 20 mM (2S,3S)-*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester (E-64d, the membrane permeable form of E-64), a general clan CA cysteine peptidase inhibitor; 10 µM N-methyl-piperazine-Phe-homoPhe-vinylsulfone-phenyl (K11777), a more specific cysteine peptidase inhibitor, acting against Clan CA, family C1; 1 µM 1,10 phenanthroline and 250 µM EDTA, both metallo-peptidase inhibitors; 2 µM pepstatin A, an aspartic peptidase inhibitor; 1 mM phenylmethyl sulfonyl fluoride (PMSF) and 100 µM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF or pefabloc), both serine peptidase inhibitors; 25 µM antipain and 50 µM leupeptin, both inhibitors of serine and cysteine peptidases; 100 µM MG132 and 100 µM lactacystin, proteasome inhibitors; 100 µg/ml cytochalasin D, an inhibitor of actin polymerization; and 100 µM pentamidine, and 1000 µM of the polyamine CMR compounds 3, 32 and 370, synthesised by Caroline Reid in the Department of Chemistry, University of Glasgow, as detailed in Section 4.2.6.

When an inhibitor was found to be effective at preventing cleavage, a serial dilution of concentrations was prepared and tested against the cells in order to determine an IC_{50} value for inhibition of Bz-R-AMC cleavage.

Fluorescence was read every min for 30 repeats, in an EnVision™ Reader (Perkin Elmer) at emission wavelength of 355 nm, excitation of 460 nm and using the general mirror. The fluorescence readings (minus the fluorescence of the control wells) were converted to a concentration of AMC released using the AMC standard. The figures were graphed using Microsoft Excel to determine the rate of cleavage of Bz-R-AMC over the 30 min time frame, with the rates converted to relative activities compared to uninhibited cells. IC_{50} values were calculated from these, using GraFit 5 data analysis software (Erithacus Software, Horley, UK).

2.5 Molecular Biology Techniques

2.5.1 Isolation of genomic DNA from *L. major*

Genomic DNA was prepared following the “cultured animal cells” protocol of the DNeasy tissue kit (Qiagen, Crawley, UK). DNA was quantified either by running out a 1 µl sample on an agarose gel and comparison to the 1 kb ladder (as in Section

2.5.4.2) or by analysing a 1 in 100 dilution of DNA using an Eppendorf Spectrophotometer. DNA concentration was calculated using the Beer-Lambert Law: concentration (µg/ml) = Abs $_{\lambda=260nm}$ x sample dilution x 50 µg/ml (where 50 µg/ml represents the concentration of DNA necessary to obtain 1 unit of absorbance at λ=260nm).

2.5.2 Polymerase chain reactions (PCRs)

All oligonucleotides were synthesised by MWG-Biotech (Ebersberg, Germany). PCR reactions were performed using either *Taq* DNA polymerase (AB gene, Epsom, UK or NEB, Hitchin, UK) or Phusion™ Hot Start high-fidelity *Taq* polymerase (NEB, Hitchin, UK). The annealing temperature and elongation time used were optimised for each reaction. A G-Storm GS4 PCR machine was used (GRI Ltd, Braintree, UK) for PCR cycles.

For reactions using *Taq* polymerase, the following concentrations of reagents were used: 2 µl of 10x PCR mix (1.13 mg/ml BSA, 450 mM Tris pH 8.8, 110 mM ammonium sulphate, 45 mM $MgCl_2$, 68.3 mM β-mercaptoethanol, 44 µM EDTA pH 8.0, 10 mM dCTP, 10 mM dATP, 10 mM dGTP, 10 mM dTTP, water), 10 pmol of each oligonucleotide, 50-100 ng of DNA and 0.5 units of *Taq* polymerase in a final volume of 20 µl. The PCR cycles were: 95℃ for 30 sec, 1 cycle; 95℃ for 30 sec, 56℃ for 30 sec, 72℃ for y min, 30 cycles; finally 72℃ for 10 min. The elongation time (y) was estimated from the size of the fragment to be amplified, based on the assumption that typically around 1 kb was synthesised per min.

For reactions using the proof-reading polymerase, the following concentrations of reagents were used: 4 µl of 5x Phusion GC buffer (NEB, Hitchin, UK), 1 mM of dNTPs mix, 10 pmol of each oligonucleotide, 50-100 ng of genomic DNA and 1 unit of Phusion high-fidelity *Taq* polymerase, in a final volume of 20 µl. The PCR cycles were: 95℃ for 30 sec, 1 cycle; 95℃ for 30 sec, 56℃ for 1 min, 72℃ for y min (as above), 35 cycles; finally 72℃ for 10 min. The annealing temperature was adjusted depending on the melting temperature of the oligonucleotides in use.

2.5.3 Oligonucleotides used

Oligonucleotides are shown in Table 2.1. Sequences are written in 5' → 3' sense. The orientations of the primers are indicated, F for forward and R for reverse. If the

oligonucleotides contain restriction sites, the restriction site is underlined, with the corresponding restriction enzyme named. For site-directed mutagenesis, the codon that has been modified is underlined and the mutated nucleotide indicated in bold.

Table 2.1 Oligonucleotides used in this study.

Oligonucleotide	Forward / Reverse	Sequence (restriction site / codon modified underlined)	Restriction enzyme
OL2352	F	A CCC GGG ATG TCG TCG GAC AGC TCC GTC	*Xma* I
OL2353	R	T AGA TCT TTA CCT GCG AAC CAG CAG GCG	*Bgl* II
OL2395	F	C AAG CTT CTC CTT CTC GGT GGC ACT TG	*Hin*d III
OL2415	R	CC GTT GGG CAC GCA TGT ACC GTC GAC G	*Sal* I
OL2356	F	G CCC GGG GAG TCG TGA ACA TTA ACT CC	*Xma* I
OL2459	R	G AGA TCT CGG TAG CGG GAG AGA GAA GG	*Bgl* II
OL2520	F	GAG CAC CTC AAC AAG GAG A**A**G GTC TAC TTC CAG GCG CGC	
OL2521	R	GCG CGC CTG GAA GTA GAC C**T**T CTC CTT GTT GAG GTG CTC	
OL2550	F	GCC TGC GAG GGG CGT **G**GC GCC GGT GGC CTG C	
OL2551	R	G CAG GCC ACC GGC GC**C** ACG CCC CTC GCA GGC	
OL1853	F	CTG GAT CAT TTT CCG ATG	
OL1854	R	TGA TAC CAC TTA TCG CAC TT	
M13 Forward	F	GTA AAA CGA CGG CCA G	
M13 Reverse	R	GGA AAC AGC TAT GAC CAT G	
T3	F	AAT TAA CCC TCA CTA AAG GG	
T7	R	TAA TAC GAC TCA CTA TAG GG	
OL2456	R	CCT CGT TAC CGC TCA TGT CC	
OL816	F	CTG TCA TCT CAC CTT GCT CC	
OL13	R	GGT GAG TTC AGG CTT TTT CA	
OL14	F	CGT CCG AGG GCA AAG GAA TA	
OL17	R	CAG GGA TCA CCG AAA TCT TCA	
OL18	F	CGG GAG CAC AGG ATG ACG CCT	
OL2615	F	CGT GCC GCA TGG CCG CCA AC	
OL2616	R	CGC CGC CGA GGG CGG GAA GG	
OL2702	F	TGA AGA TTT CGG TGA TCC CTG	
OL2703	R	AGG CGT CAT CCT GTG CTC CCG	
OL2704	F	TGA AAA AGC CTG AAC TCA CC	
OL2705	R	TAT TCC TTT GCC CTC GGA CG	
OL2399	F	CAC GGT GCA CCT CGT GGA GTC	

2.5.4 Cloning of DNA fragments

Cloning of DNA fragments used two strategies. One method involved digestion by restriction enzymes of both DNA insert and vector recipient, followed by ligation of the fragments. The second involved cloning of PCR products by amplication using either *Taq* polymerase or Phusion high-fidelity *Taq* polymerase. *Taq* polymerase adds a single A residue to the 3' ends of the PCR product, which can then be inserted into the pCR2.1-TOPO vector (Invitrogen, Paisley, UK). Phusion polymerase does not add the single A and these fragments can thus be inserted into the PCR-Script Amp cloning vector (Stratagene, La Jolla, CA, USA).

2.5.4.1 Digestion of DNA with restriction enzymes

Typically, restriction digests used 5 units of restriction enzyme and 1x buffer in a total volume of 20 µl per 500 ng of DNA to be digested. The buffer used was provided with the enzyme by the manufacturer (NEB, Hitchen, UK). For digests using multiple enzymes, compatible buffers were chosen. The reactions were incubated at the temperature specified by the manufacturer for 1-4 h on average.

2.5.4.2 DNA gel electrophoresis and gel extraction

Analysis was performed using 1% (w/v) agarose gels (Invitrogen, Paisley, UK), in 0.5 x TBE (20 mM Tris, 20 mM boric acid, 0.5 mM EDTA pH 7.2). Sybr safe™ DNA gel stain (Invitrogen, Paisley, UK) was added to the agarose gel, at a 1x concentration, to allow the visualisation of the DNA by exposure to UV light (Gel Doc 2000 (BioRad)). A 1 kb ladder molecular weight marker (Invitrogen, Paisley, UK) was used at a concentration of 1 µg per lane to determine the size and concentration of the analysed DNA (at this concentration the 1.6 kb marker band is composed of 100 ng of DNA, therefore, the concentration of fragments of comparable size and intensity are similar). For PCR sample analysis, 10 µl of the reaction were mixed with 2 µl of 6x DNA loading buffer (50% (v/v) glycerol, 0.25% (w/v) bromophenol blue in TBE).

Following PCR reactions or enzymatic digestion, DNA of interest was isolated and purified using the Gel Extraction Kit (Qiagen, Crawley, UK) as specified in the manufacturer's instructions. To be concentrated or when used for transfection, the DNA was ethanol-precipitated by addition of 2 volumes of 100% ethanol and

10% 3 M NaAc pH 5.2 and centrifugation at 18,000 *g* for 30 min. The DNA pellet was washed twice with chilled 70% ethanol. Once air-dried, the pellet was re-suspended in a suitable volume of sterile water and kept at -20℃ until required.

2.5.4.3 Ligations

PCR products generated, as described previously with the *Taq* DNA polymerase or Phusion high-fidelity Taq polymerase, were ligated into the pCR2.1-TOPO vector or pPCR-Script vector, respectively, following the manufacturer's instructions.

For ligation into the final cloning vector, gel-purified digested DNA fragments and 100-200 ng of gel-purified digested vector were mixed together at a molecular ratio of 7:1 and incubated with 40 units of T4 DNA ligase and 1 µl of 10x T4 DNA ligase buffer (both NEB, Hitchin, UK) in a final volume of 10 µl, either overnight at 16℃ or for 1 h at room temperature.

These ligations were used to transform heat-shock competent *Escherichia coli* XL-1 blue cells (see Section 2.5.4.5) and transformants were selected on Luria-Bertani (LB) agar (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 20% (w/v) agar) plates supplemented with the appropriate antibiotics.

2.5.4.4 Competent cells

E. coli XL-1 blue cells were plated on an LB agar plate with tetracycline. One colony was used to inoculate 10 ml of LB medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl) and incubated overnight at 37℃. The overnight culture was diluted 1:100 in 50 ml of LB medium and grown at 37℃, until an OD=0.7 (at 600 nm). The cells were transferred to a 50 ml tube and put on ice for 10 min before centrifugation at 350 *g* for 15 min. The cell pellet was resuspended in 16 ml of RF1 buffer (100 mM rubidium chloride, 50 mM $MnCl_2.4H_20$, 30 mM potassium acetate, 10 mM $CaCl_2$, 15% glycerol, with a final pH of 5.8, adjusted with 0.2 M acetic acid) and incubated on ice for 15 min. The suspension was centrifuged for 15 min at 540 *g* at 4℃. The cell pellet was resuspended, with careful pipetting, in 4 ml of RF2 buffer (10 mM MOPS pH 6.8, 10 mM rubidium chloride, 75 mM $CaCl_2$, 15% glycerol, with a final pH of 6.8, adjusted with NaOH) and incubated on ice for 1 h. Heat-shock competent cells were then aliquoted and deep-frozen with dry ice, with aliquots subsequently stored at -80℃.

2.5.4.5 Transformation of competent cells

Frozen heat-shock competent *E. coli* cells were placed on ice to defrost. The DNA of interest was added to the cells and incubated with them, still on ice, for 30 min. Transformation was performed by incubation of the cells-DNA mix at 42℃ for 45 sec, following which the suspension was placed on ice for a further 2 min, 100 µl of LB medium was added and the mixture was spread on LB plates, containing the relevant antibiotic. The plates were incubated overnight at 37℃.

2.5.4.6 Colony screening by PCR

Bacterial colonies were taken from their plate with a sterile 200 µl pipette tip, streaked onto a new LB Agar plate and residual bacteria dipped into a *Taq* DNA polymerase mediated PCR reaction (made up as detailed in Section 2.5.2). For screening the presence of insert in the pPCR-Script or TOPO vector, the T3/T7 and M13F/M13R oligonucleotide couples were used, respectively. When using different plasmids, the relevant oligonucleotides to be used were determined using the Vector-Nti map of the constructs and the “research motif” function of this software (Vector-Nti Advance 10, Invitrogen). Only primers with a level of homology higher than 80% were used.

PCR products were analysed on an agarose gel as detailed in Section 2.5.4.2. Clones of interest were selected and the relevant colony was picked from the plate and used to inoculate 5 ml of LB medium. After an overnight incubation at 37℃, 600 µl of the culture was mixed with 600 µl of a 2% (w/v) peptone, 40% (v/v) glycerol solution and placed at -80℃ for cryopreservation and long term storage.

2.5.4.7 Plasmid DNA purification

The bacterial culture of the clones identified by screening were processed with a MiniPrep kit (Qiaspin miniprep, Qiagen, Crawley, UK), following the manufacturer’s instructions, to extract the plasmid DNA. The plasmid DNA was eluted in water and stored at -20℃ until needed. When a larger amount of DNA was required, for example for transfection of *Leishmania* cells, 100 ml of bacterial culture was grown up overnight in LB medium at 37℃ and the DNA was extracted using either several miniprep columns or a MidiPrep kit (Qiagen, Crawley, UK). The DNA was then ethanol-precipitated.

2.5.4.8 Plasmid generation

DNA inserts were transferred between vectors by using single-cutter restriction enzymes. To enable this, the necessary restriction sites were added to the 5' and 3' ends of the oligonucleotides used for PCR amplifications. Therefore, after a DNA fragment was sequenced to confirm its conformity, it could be easily cut from the sub-cloning vector by enzymatic digestion. Restriction enzymes (NEB, Hitchin, UK) were used at 10 units per µg of DNA in the appropriate reaction buffer (NEB, Hitchin, UK), following the manufacturer's recommendations.

Most of the enzymes used generated 5' or 3' overhangs. However, when, during the preparation of the vector DNA, there was no other possibility than to use restriction enzymes creating blunt-ended fragment, the calf intestinal alkaline phosphatase (CIP; NEB) was used, as indicated by the manufacturer, to dephosphorylate the digested DNA and prevent the linearised plasmid from religating to itself.

In order to blunt the ends of fragments, Mung bean Nuclease (NEB) was used after digestion to degrade single-stranded extensions from the end of the DNA.

The plasmids generated in this study are detailed in Table 2.2 and further discussed in Chapter 5.

Table 2.2 Plasmids generated in this study.

Plasmid number	Purpose
pGL1640	Over-expression of WT OPB
pGL1714	Over-expression of active site mutant OPB
pGL1693	Deletion of OPB – with Hygromycin B phosphotransferase
pGL1762	Deletion of OPB – with Streptothricin acetyltransferase

2.5.5 DNA sequencing and analysis

DNA sequencing was performed by the Sequencing Service, University of Dundee (www.dnaseq.co.uk/), using an ABI 3730 capillary DNA sequencer. Electro-phoregrams were then edited and compiled into contigs using ContigExpress and Align X (modules of Vector Nti Advance™ 10; Invitrogen, Paisley, UK).

2.5.5.1 Sequence alignments

Amino acid sequences were imported either from the GeneDB (www.genedb.org) or the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov/) websites into Vector NTI Advance 10. Alternatively, the amino acid sequences were obtained from translation of nucleotide open reading frames (in Vector NTI Advance 10). Alignments were performed using the ContigExpress module of the Vector NTI Advance 10 package.

2.5.6 Site-directed mutagenesis

Site directed mutagenesis was used to correct a single base alteration in the *L. major* OPB over-expression construct, pGL1640, and to introduce a site-specific mutation in the active site of *L. major* OPB in order to generate an inactive OPB over-expression construct, pGL1714. The mutation chosen altered the active site serine to glycine (S577G), with a single point-mutation required, using pGL1640 as the template.

To correct the single base alteration in pGL1640 the oligonucleotides OL2520 and OL2521 were used, whilst to generate pGL1714 the oligonucleotides OL2550 and OL2551 were used.

The QuikChange Site-Directed Mutagenesis Kit was used (Stratagene, La Jolla, CA, USA). The reactions contained 1x reaction buffer (provided with the kit), 50 ng of plasmid DNA (plasmid carrying the gene to mutate), 125 ng of each forward and reverse primer, 1 µl of dNTP mix and 2.5 units of *Pfu Turbo* DNA polymerase. The following cycle was used: 95℃ for 1 min, then 18 cycles of 95℃ for 30 sec, 60℃ for 1 min and 68℃ for 8 min, followed by 10 min at 68℃. Ten units of *Dpn*I enzyme were added to the completed reaction and this was incubated at 37°C overnight, to remove any residual methylated template. 90 µl of *E. coli* XL-1 Blue supercompetent cells were transformed with 10 µl of digested PCR using the heat shock method (see 2.6.4.5) and were plated on LB agar plates. The plates were incubated overnight at 37℃. Plasmid was extracted from colonies, sequenced and used for *Leishmania* transfection.

2.5.7 Transfection of *Leishmania*

2.5.7.1 Plasmid DNA Preparation

For integrative constructs, 45 µg of plasmid was digested with the appropriate restriction enzymes (see Figures 5.8 and 6.1 for details), separated by gel electrophoresis and gel extracted, as described in Section 2.5.4.2. The digested DNA was precipitated with ethanol. For ectopic constructs, 20 µg of circular plasmid was directly precipitated with ethanol. In both cases, the last washing step of the precipitation was done in a sterile Category II cabinet. The precipitated DNA was resuspended in 20 µl of sterile water and used for transfection of 5 x 10^7 *L. major* promastigotes.

2.5.7.2 Electroporation and cloning

Promastigotes from mid-log phase cultures, at a density of around 1-1.5 x 10^7 cells/ml were used. 5 x 10^7 *L. major* and 10-20 µg of purified (digested or not) plasmid DNA (in 20 µl water) were necessary for each transfection. Two methods were used. For the first, cells were counted and the appropriate number were centrifuged for 10 min at 1000 *g* at 4℃ and washed with ElectroPoration Buffer (EPB, 120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4, 25 mM HEPES, 2 mM EDTA, 2 mM $MgCl_2$ at pH 7.6). Cells were resuspended at 1×10^8 cells/ml in 500 µl EPB and incubated with the plasmid DNA, in an ice-cold 4-mm gap cuvette (Gene Pulse, BioRad, Hemel Hempstead, UK) on ice for 10 min. Electroporation was done twice at 25 µFarads, 1500 Volts (3.75kV/cm), pausing 10 sec between pulses, using a BioRad Gene Pulser II machine.

For the second method, cells were counted, the appropriate number were centrifuged for 10 min at 1000 *g* at 4℃ and 5×10^7 cells were resuspended in 100 µl of Nucleofector Solution from the Amaxa Human T Cell Nucleofector Kit (Amaxa AG, Cologne, Germany). These were mixed with the plasmid DNA, in a cuvette from the Nucleofector kit. Program U-033 was used to electroporate the cells using an Amaxa Nucleofector Device.

In both cases, the transfected cells were stored on ice for 10 min, then transferred to a 25 cm^3 phenolic-style lid flask containing 5 ml of HOMEM medium with

20% (v/v) HIFCS and left to recover overnight at 25℃. Antibiotic selection of successful transfectants was started the following day.

To generate a population of cells, for ectopic expression, antibiotics were added directly to the flask of cells (concentrations used are described in Section 2.1.1). To generate individual clones, transfected cells were diluted in three steps. This method is based on the calculated transfection efficiency in *Leishmania* in order to dilute the promastigotes to a concentration which will give an average of 0.1 cells/ well in the middle dilution. Firstly 4 ml of cells was added to 20 ml of HOMEM with 20% (v/v) HIFCS and the relevant antibiotics and mixed. 2 ml of this dilution was then added to 22 ml of HOMEM with 20% (v/v) HIFCS and the relevant antibiotics and mixed. 2 ml of the second dilution was then added to a further 22 ml of HOMEM with 20% HIFCS and the relevant antibiotics and mixed. Each dilution was plated out in a 96 well plate, with one plate per dilution and 200 µl per well. Genomic DNA was extracted from drug resistant populations and clones and presence of the ectopic construct or correct integration was checked by PCR.

2.5.8 Species Check of *Leishmania* by PCR

Genomic DNA was extracted for the parasites to be checked. The method used was based on that of Schonian et al. (Schonian et al., 2003). A PCR reaction was completed by the method in Section 2.5.2 using primers OL1853 and OL1854 and the genomic DNA of interest and standard DNA of known species. The PCR products obtained were digested with *Hae* III enzyme (Section 2.5.4.1) and then the digested DNA was visualised by agarose gel electrophoresis (Section 2.5.4.2) and the samples of interest compared to the digestion pattern of the known standards to enable identification of the species.

2.6 Biochemical Methods

2.6.1 SDS-PAGE

Cell lysates were mixed with equal volumes of 2x loading buffer and denatured at 100℃ for 5 min. Protein separation was carried ou t using the polyacrylamide gel method described by Laemmli (1970). Gels used 12% (w/v) polyacrylamide (Bio-Rad, Hemel Hemstead, UK) and were run using a Mini-Protean II system (Bio-Rad), according to the manufacturer’s instructions. Gels were stained by

immersion in a solution of 0.25% (w/v) Coomassie Brilliant Blue R250, 45% ethanol, 9.2% acetic acid for 1 h at room temperature and destained using several changes of destain solution (10% (v/v) acetic acid, 12.5% (v/v) methanol). Gels were then washed in water and a picture captured using a Gel Doc 2000 (BioRad). Alternatively, gels were used for immunodetection (western immunoblot).

2.6.2 Antibodies

2.6.2.1 Production

Full length recombinant OPB protein was produced in *E. coli* (by Dr. Gareth Westrop, University of Glasgow) and used to raise polyclonal antiserum in a sheep using standard procedures.

2.6.2.2 Affinity Purification

OPB antiserum was affinity purified using a 5 ml polypropylene column (Pierce, Cramlington, UK). 5 mg of recombinant OPB was covalently linked to 500 µl of Aminolink Coupling Gel (Pierce), following the manufacturer's protocol. The OPB/ aminolink resin was then equilibrated with 10 ml of IgG binding buffer (0.14 M NaCl, 8 mM sodium phosphate buffer pH=7.5, 2 mM potassium phosphate buffer pH 7.5, 0.01 M KCl). 2 ml of anti-OPB sheep antiserum (final bleed) was then diluted by half in IgG binding buffer and incubated overnight at 4℃ with the protein/aminolink resin. The resin was washed with 12 ml of IgG binding buffer before eluting the bound antibodies with 2.5 ml of Immunopure IgG elution buffer (Pierce).

A PD-10 Desalting column (GE Healthcare, Chalfont St Giles, UK) was used to change the buffer from IgG elution buffer to PBS, as per the manufacturer's instructions. The purified antibodies were stored at -20℃ in aliquots.

2.6.3 Western immunoblotting

SDS-PAGE gels were first incubated for 10-15 min in transfer buffer to equilibrate (20 mM Tris/HCl, 15 mM glycine, 20% (v/v) methanol). Separated proteins were transferred by electrophoresis to Hybond P (PVDF) membrane (Amersham, Chalfont St Giles, UK) using the Mini Protein II transblot module at 100 V for 1 h at room temperature or at 30 V overnight at 4℃. The membrane was blocked for 1 h

at room temperature in PBS-T (0.1% Tween-20 in 1x PBS) with 5% (w/v) low fat dried milk powder, then incubated in fresh blocking buffer containing the appropriate primary antibody at room temperature for 1 h or overnight at 4℃, both with constant, gentle agitation.

The affinity purified sheep antibody against OPB was used at a 1:20,000 dilution and monoclonal anti-luciferase antibody (Calbiochem, Nottingham, UK) was used at 1:1000. Monoclonal anti-GP63 antibody (Button et al., 1991), was used at 1:100. Mouse anti-TbEF1α antibody (Upstate, Chandlers Ford, UK), was used at 1:10,000. Rabbit anti-HASPB antibody (Flinn et al., 1994) was used at 1:1000.

The membranes were washed briefly twice in PBS-T, to remove the majority of the antibody-milk solution, and then a further 4x in PBS-T, each for 15 min. After this, they were incubated with secondary antibody for 1 h at room temperature in PBS-T plus 5% (w/v) low fat milk. Depending on the primary antibody used, secondary antibodies were goat anti-mouse, donkey anti-sheep or goat anti-rabbit Horse Radish Peroxidase (HRP)-conjugated antibodies, all at 1:5000 dilution. Following incubation with secondary antibody, membranes were washed briefly twice in PBS-T, to remove the majority of the antibody-milk solution, and then a further 4x in PBS-T, each for 15 min. The membrane was then incubated with an ECL kit (SuperSignal West Pico Chemoluminescent Substrate, Pierce, Cramlington, UK) for 5-15 min and were either subsequently exposed to autoradiography film for various time intervals and developed using a Kodak M35-M Xomat Processor (Kodak, Hemel Hemstead, UK), or were visualised using a Molecular Imager ChemiDoc XRS System (Bio-Rad, Hemel Hemstead, UK).

2.6.4 Immunoprecipitation

L. major cells were lysed by sonication (see Section 2.1.6) in Binding Buffer A (0.1 M sodium phosphate buffer, 0.15 M NaCl, pH 7.4) with the following inhibitors: E-64, pepstatin A, 1,10 phenanthroline, PMSF and EDTA (at the concentrations described in Section 2.1.6). The lysate was filter sterilised and incubated overnight at 4℃ in a 50:50 mix of either lysate:un purified anti-OPB antibody or lysate:pre-immune serum.

A ProPur Protein G Mini Spin kit (Nunc, Roskilde, Denmark) was used. The manufacturer’s instructions were followed, using the buffers Elution Buffer B2

(0.2 M glycine/HCl buffer, pH 2.5) and Neutralisation Buffer C (1 M Tris/HCl buffer, pH 9.0). A sample was taken of the lysate mixtures that went on the column, and for both mixtures the following samples were collected: flow through from column, three washes and two elutions.

The samples were used in a Bz-R-AMC degradation assay (as detailed in Section 2.4.1) and were run on an SDS-PAGE gel and transferred for a western immunoblot (Sections 2.6.1 and 2.6.3).

2.6.5 Immunofluorescence Analysis

2.6.5.1 Fixation of promastigote *Leishmania*

Leishmania promastigotes were washed twice in PBS, then fixed in 200 µl of 1% formaldehyde/PBS for 30 min at room temperature. The cells were permeabilised by adding 20 µl of 1% Triton X-100/PBS for 10 min and 20 µl of 1M glycine/PBS was added to neutralise free aldehyde bonds that resulted from the formaldehyde fixation, to diminish any background fluorescence, and incubated a further 10 min.

During these incubations, glass slides were washed with 70% ethanol. A small rectangular border of clear nail varnish was drawn onto the slide to limit the spread of cells and antibody solutions. The slides were then coated with 20 µl poly-L-lysine (Sigma-Aldrich, Poole, UK) and air dried. The treated cells were pipetted onto these slides and left to sediment and adhere to the surface for 15-30 min in a dark box containing PBS-soaked tissues, to prevent drying.

2.6.5.2 Infection and fixation of PEM

PEM were extracted from ICR mice as described in Section 2.2.1. 13 mm diameter coverslips (VWR International, Leicestershire, UK) were cleaned with 70% ethanol and one placed at the bottom of the appropriate number of wells of a 12 well plate. 500 µl of PEM at 5×10^5 cells/ml was added to each well. Cells were left to adhere overnight at 37℃ with 5% CO_2. PEM were generally infected with *Leishmania* the day following extraction, but they could be left for up to one week prior to infection.

PEM were infected with cultured promastigotes at a 7:1 ratio for *L. major* and incubated at 37℃. Parasites were left to infect o vernight, then the wells were washed twice with RPMI 1640 and the macrophages were re-covered with 1 ml RMPI 1640 with 10% (v/v) HIFCS. THP-1 cells, differentiated as in Section 2.1.7 were infected and treated as PEM for immunofluorescence.

Following the desired number of days, PEM were washed twice in PBS and then fixed by addition of 500 µl of 1% formaldehyde/PBS to each well for 30 min at room temperature. The cells were permeabilised by adding 50 µl of 1% Triton X-100/ PBS for 10 min, then 50 µl of 1M glycine/PBS was added and incubated a further 10 min. The wells were washed twice with PBS prior to antibody addition.

2.6.5.3 Immunofluorescence

The primary antibody was diluted in 0.1% Triton X-100, 0.1% BSA in PBS (TB), added to the slide / well and incubated with the cells for at least 1 h at room temperature. They were then washed with 10 ml of PBS. The rhodamine (red) – conjugated secondary antibody (Calbiochem, Nottingham, UK) was diluted at 1/1000 in TB and then added and the slide / well were incubated in the dark for 1 h at room temperature. 1 µg/ml of 4,6-diamidino-2-phenylindole (DAPI; Sigma, Poole, UK) was added for 1 min before proceeding to a 10 ml PBS wash.

For promastigotes, as much liquid as possible was removed, as well as the nail varnish border, and a mounting solution (2.5% DABCO, 50% glycerol in PBS) was applied to the coverslip before its application to the slide. The coverslips with attached PEM were lifted from their wells and applied cell-side down to a slide. Coverslips were sealed with nail varnish to prevent drying out.

The fluorescence was observed with the “DAPI” (λ = 457 nm), and “Rhodamine” (λ = 617 nm) filters of the UV light microscope. Cells were viewed with an Applied Precision DeltaVision microscope and SoftWoRx Suite software (Image Solutions Ltd, Preston, UK). Images were prepared using Adobe Photoshop CS.

2.6.5.4 Colocalisation of Concanavalin A with immunofluorescence

Concanavalin A (Molecular Probes, Paisley, UK) was made up to 5 mg/ml stock as per manufacturer’s instructions. 500 µl of *Leishmania* cell suspension was

centrifuged for 5 min at 1000 *g* at room temperature, washed with HOMEM medium and resuspended in 500 µl of HOMEM containing 5 µg Concanavalin A and incubated for 1 h. The cells were washed twice in PBS and resuspended in PBS. The protocol for immuno-fluorescence was then followed with the stained cells from the fixation stage (Sections 2.6.5.1 and 2.6.5.3). The fluorescence was observed with the "FITC" (λ = 526 nm) filter of the UV light microscope.

2.7 Fluorescent microscopy

2.7.1 Assessment of Bz-R-AMC uptake into *L. major*

Bz-R-AMC was made up at a stock concentration of 100 mM as per the manufacturer's instructions. 1 ml of cell suspension was centrifuged for 5 min at 1000 *g*, washed twice with HOMEM medium, resuspended in 500 µl of HOMEM with 50 µM Bz-R-AMC and incubated for 10 min. The cells were washed three times in PBS and resuspended in PBS. 20 µl of poly-L-lysine (Sigma-Aldrich, Poole, UK) was spread on a Twin frosted 76x26 mm microscope slide (VWR International, Lutterworth, UK) and left to dry. The cells were spread on this and a 22x64 mm coverslip (VWR International) was fixed over them with clear nail varnish.

The fluorescence was observed with the AMC filter of the Zeiss UV microscope, with an exposure time of 250 ms. Phase was observed against bright visible light and pictures were taken with an exposure time of 50 ms. Images were captured by an Orca-ER camera (Hamamatsu Photonics, Welwyn Garden City, UK) and Openlab software version 4.0.3 (Improvision, Coventry, UK) and prepared for presentation using Adobe Photoshop CS.

2.7.2 Assessment of FM4-64 uptake

1 ml of cell suspension was centrifuged for 5 min at 1000 *g*, washed twice with HOMEM medium, resuspended in 1 ml of HOMEM either with or without 100 µM cytochalasin D and incubated for 15 min, prior to the addition of 40 µM FM4-64 (from a 12 mM stock solution in DMSO; Invitrogen) and a further 10 min incubation at 4℃. The cells were washed three times in cold PBS and resuspended in PBS. 20 µl of poly-L-lysine (Sigma-Aldrich) was spread on Twin frosted 76x26 mm microscope slides (VWR International) and left to dry. Samples of both inhibited

and uninhibited cells were spread on these slides and a 22x64 mm coverslip (VWR International) was fixed over them with clear nail varnish.

The fluorescence was observed with the “Rhodamine” filter of the Applied Precision DeltaVision microscope and SoftWoRx Suite software (Image Solutions Ltd). Images were prepared for presentation using Adobe Photoshop CS.

2.8 DNA Content Analysis

2.8.1 Cell preparation

Mid-log phase promastigotes were centrifuged at 1000 *g*, 4℃ for 5 mins, washed with PBS, centrifuged again and finally resuspended in 1 ml of 70% methanol in PBS. Cells were stored at 4℃ for at least an hour or overnight. Prior to analysis, the cells were centrifuged at 1000 *g*, 4℃ for 5 min, washed with 1 ml PBS once, centrifuged, resuspended in 1 ml of PBS with 10 µg/ml propidium iodide and 10 µg/ml RNAse A (both Sigma-Aldrich, Poole, UK), and finally incubated at 37℃ for 1 h, in the dark.

2.8.2 Fluorescence Activated Cell Sorting (FACS) analysis

FACS analysis was performed with a Becton Dickinson FACSCalibur using the FL2-A (fluorescence intensity at 585/642 nm, note $\lambda_{emission}$ propidium Iodine = 620 nm under an $\lambda_{excitation}$ = 488 nm (blue, Uniphase Argon Ion laser)), the Forward Scatter (FSC, relative cell size) and the Side Scatter detectors (SSC, cell granulometry or internal complexity). Introduction of the prepared samples into the cytometer was automated with a FACS Loader (Worklist manager and Loader manager softwares). For each sample, 10 000 events (cells) were analysed. Data was interpreted using the CellQuestPro software (BD Bioscience, Oxford, UK).

2.9 Analysis of Luciferase Activity

2.9.1 Generation of luciferase expressing *Leishmania*

Two constructs were used to generate luciferase-expressing *Leishmania*; one for integration into the *Leishmania* genome and the other for ectopic expression.

The integrating construct was a gift of Prof. D.F. Smith (University of York, UK). It was designed to integrate into the ribosomal RNA small subunit of *Leishmania*. The construct was derived from the pSSU-int construct of Dr T. Aebischer of the University of Edinburgh, UK (Misslitz et al., 2000). The vector, pGL1313 (illustrated in Figure 6.1), was prepared for transfection by enzymatic digestion and transfected into *L. major, L. mexicana* and *L. infantum* promastigotes, as described in Section 2.5.7. Two clones were selected for each species.

The ectopic construct was a gift of Prof. M. Ouellette (McGill University, Montreal, Canada). The vector map is shown in Figure 6.2. The ectopic construct was transfected into *L. mexicana, L. major* and *L. infantum* promastigotes (as described in Section 2.5.7), and the promastigotes maintained as populations.

Further details of both sets of luciferase lines are given in Section 6.2.1.

2.9.2 Using D-luciferin with promastigote *Leishmania*

Leishmania promastigotes were counted and a serial dilution of cells was completed, with parasites diluted to the relevant concentration of cells per ml in phenol red free RPMI 1640 (PAA Cell Culture, Yeovil, UK). 180 µl of cell suspension was pipetted into the wells of a 96 well plate to which 20 µl of a 1 mM stock solution of D-luciferin (Promega, Southampton, UK) was added, giving a final concentration of 12.5 µM D-luciferin. One or two replicates were completed for each dilution of cells per experiment. Luminescence was read using an EnVision™ Multilabel Reader (Perkin Elmer), with Luminescence mirror and Luminescence <700 emission filter.

2.9.3 Using luciferase assay kit with promastigote *Leishmania*

2.9.3.1 Using *Leishmania* with integrated luciferase construct

Leishmania promastigotes were counted and a serial dilution was completed, with promastigotes diluted to the relevant concentration in phenol red free RPMI 1640. 100 µl of promastigote suspension was pipetted into the wells of a 96 well plate and 100 µl of the assay solution from the Luciferase Reporter Gene Assay, constant light signal kit (Roche, Burgess Hill, UK) was added. A single replicate was completed per experiment for each concentration of cells, with three experiments completed. Luminescence was read using an EnVision Reader, as described in Section 2.9.2.

2.9.3.2 Using *Leishmania* with ectopic luciferase construct

Leishmania promastigotes were counted and made up to 2 x 10^7 cells/ml in phenol red free RPMI 1640. 150 µl of promastigote suspension was pipetted into the wells of a 96 well plate, with 50 µl of the assay solution from the Luciferase Reporter Gene Assay, constant light signal kit was added. Two or three replicates were completed per experiment. Luminescence was read using an EnVision plate reader, as described in Section 2.9.1.

2.9.4 Using luciferase assay kit with infected PEM

PEM were harvested as described in Sections 2.2.1 and 100 µl of 5 x 10^5 /ml cell suspension was added to either the wells of 96-well plates, or to 16-well slides for control experiments, and they were incubated at 37°C, 5% CO_2 overnight. PEM were infected with stationary phase cultures of luciferase-expressing promastigotes as described in Sections 2.2.2. PEM were infected at a 5:1 ratio of promastigotes:PEM.

The slides were incubated at 37℃ with 5% CO_2 for 2-3 days, with the un-engulfed promastigotes washed off at the 24 h time point and fresh RPMI 1640 with 10% (v/v) HIFCS added.

Following incubation, the wells were washed twice with phenol red-free RPMI 1640 medium. The wells of the 96-well plate had 100 µl of phenol red-free RPMI 1640 added and 100 µl of the assay solution from the Luciferase Reporter Gene Assay, constant light signal kit. Plates were read in the EnVision plate reader, as described in Section 2.9.1. The 16-well slides were fixed with 100% methanol for around 1 min and stained with 10% Giemsa's stain for 10 min and the percentage of macrophages infected was determined by light microscopy.

2.10 Statistical Analysis

Values were expressed as means ± standard error of the mean (SEM). Levels of significance were calculated by unpaired t tests using the Data Analysis add-on of Microsoft Excel. Differences were considered significant at a p value <0.05.

Chapter 3

THE USE OF A FLUORESCENT SUBSTRATE TO CHARACTERISE PEPTIDASE ACTIVITY IN LIVE *LEISHMANIA*

3.1 Introduction

For the investigation of peptidases within cells an ability to accurately quantify their activity in an *in vivo* situation is highly desirable. In order to do this, the catalysis of fluorescent molecules is commonly used (Boonacker and Van Noorden, 2001), either endogenous fluorescent molecules, such as NADH or FAD, or, more commonly, synthetic fluorogenic substrates. In order to detect changes in fluorescence, microscopy, flow cytometry or fluorometers have been used (Harris et al., 2000; Boonacker and Van Noorden, 2001).

3.1.1 Analysis of peptidases using fluorescent molecules

Analyses based on various fluorescing constituents have been developed and examples of their use will be described both for mammalian peptidases and *Leishmania* peptidases. A number of properties are useful for peptide substrates to be used in living cells, including: gaining access to the location of the enzyme without damage to the cell; being selectively converted by the enzyme; having sufficiently strong fluorescence upon release for ease of detection; accumulating at the site of release, rather than diffusing from the site; and being non-toxic to the cell (Boonacker and Van Noorden, 2001).

3.1.1.1 Fluorescent molecules commonly used

Many fluorochromes have been used for analysis of peptidase activity, including various coumarin-, rhodamine-, fluorescein- and cresyl violet-containing substrates (Boonacker and Van Noorden, 2001), *ortho*-amino-benzoyl substrates (Alves et al., 2001a; Judice et al., 2004; Juliano et al., 2004) and 4-methoxy-naphthylamide substrates (Valdivieso et al., 2007). Their different excitation and emission wavelengths can be valuable for analysis of several peptidases simultaneously. The substrate generally consists of a peptide joined to a leaving group, which either only fluoresces once free of the amino acids or whose emission wavelength changes once released. The specificity for a particular peptidase is mostly provided by the amino acid sequence, through its interaction with the enzyme active site, although the chemical properties of the fluorophore can also be important, particularly due to steric hindrances preventing binding in the ligand binding pocket (Boonacker et al., 2003).

3.1.1.2 Fluorescent molecules for study of mammalian peptidases

Coumarin-containing compounds are some of the most widely used fluorophores and have been often used to determine mammalian peptidase activities, though more regularly for study of peptidases *in vitro* rather than in live cell assays. As an example of a live cell assay, a 7-amino-4-chloromethylcoumarin (ACMC) linked calpain substrate was used for the determination of calpain activity in Rat-1 fibroblasts and HL60 myelomonocytes. This substrate fluoresces at a different wavelength when cleaved, allowing simultaneous measurement of the substrate and product and the use of flow cytometry to measure the rate of degradation of the substrate (Niapour and Berger, 2007). The specificity of the substrate was confirmed as inhibition of activity was only achieved using calpain inhibitors. The use of flow cytometry was advantageous as the calpain activity could be measured in several cell types simultaneously by co-staining with cell-specific markers.

A related compound, 7-amino-4-carbamoylmethylcoumarin (ACC), has been linked to a variety of peptides to evaluate the substrate specificities of a number of recombinant peptidases. A fluorogenic library was prepared with ACC linked to both a peptide and a solid resin support. The optimal P_1 amino acid for a series of cysteine peptidases and serine peptidases was determined, followed by the optimal P_2, P_3 and P_4 amino acids (Harris et al., 2000). 7-amino-4-methylcoumarin (AMC)-linked peptides have been used as substrates for recombinant cathepsins K, L, B, S, F, V, H, C and Z to assay the IC_{50} of two cathepsin inhibitors (Falgueyret et al., 2004).

A method has also been developed using two fluorophores in conjunction, 7-amino-4-fluorocoumarin (AFC) and rhodamine 110, to selectively detect peptidase activity in intact, viable cells or non-viable cells with a loss of membrane integrity, respectively, for use as a measure of cytotoxicity following treatment of cells (Niles et al., 2007). Using cultured mammalian cells, the substrate preferences of live and lysed cell peptidases were determined with a variety of peptides linked to the fluorophores. Peptidase inhibitors were used to further evaluate the peptidase activity in both live and lysed cell fractions. The ability to differentiate live cells from lysed cells was found to correlate with other measures of cytotoxicity.

In contrast to coumarin-based substrates, rhodamine-based substrates have the disadvantage that the leaving group tends to accumulate in the mitochondria

following cleavage (Van Noorden et al., 1997). Rhodamine substrates are also bi-substituted, meaning two peptides must be cleaved for full fluorescence of the molecule to be exhibited; the mono-substituted form is only intermediately fluorescent (Boonacker and Van Noorden, 2001).

Measurement of the activity of two enzyme groups has been completed using laser scanning cytometry, which allows for repeated measurement of single cells as identified by their co-ordinates on a microscope slide (Bedner et al., 1998). Di-(leucyl)-rhodamine 110 was used for the determination of the activity of L-aminopeptidases in HL-60 cells and human peripheral blood cells, whilst fluorescein diacetate was used to determine the activity of esterase in HL-60 cells. Individual cells were found to have wide variation in L-aminopeptidase and esterase activities, with subpopulations of cells in peripheral blood distinguishable by their varying enzymatic activities. The determination of esterase activity was hampered by the fluorescence of the released fluorescein fading upon repeated measurement of individual cells, meaning fluorescein diacetate was less suitable for this method.

Cresyl violet has been used since the 19th Century as a histology stain and recently was developed to act as a fluorescent leaving group for analysis of peptidase activity (Boonacker and Van Noorden, 2001). Cresyl violet rapidly diffuses from its site of release in lysed cells, so it is dependent on intact cells to be useful (Van Noorden et al., 1997). As both conjugated cresyl violet and the free molecule are fluorescent, but at differing wavelengths, the substrate and product can be simultaneously measured (Boonacker et al., 2003). Cresyl violet has been used to investigate the activity of dipeptidyl peptidase IV (DPPIV) in rat hepatocytes. In this case, the substrate was $[Ala\text{-}Pro]^2$-cresyl violet and DPPIV was localised using confocal microscopy with its activity measured using flow cytometry (Van Noorden et al., 1997). This cresyl violet-containing substrate has also been compared with another fluorescence releasing substrate, Ala-Pro-Rhodamine 110, for measurement of the activity of DPPIV (Boonacker et al., 2003). Using Jurkat cells, which have DPPIV-like peptidases but not DPPIV itself, and CD26/DPPIV-transfected Jurkat cells, the specificity of the two fluorophores were compared. Ala-Pro-rhodamine 110 was found to be cleaved by peptidases other than DPPIV, whereas $[Ala\text{-}Pro]^2$-cresyl violet was specific to DPPIV. This illustrates that the choice of fluorescent leaving group can be crucial for determining the specificity of a substrate (Boonacker et al., 2003).

Cresyl violet has also been used for the measurement of cysteine cathepsin activity in mouse splenic B and T cells and J774 macrophage cells (Creasy et al., 2007), with flow cytometry used to detect the activity. The cresyl violet was bi-substituted with a peptide sequence selected for the cathepsin under study. Such peptides were used to measure the activity of cathepsins B, L and S, with cathepsin specific inhibitors used to confirm the cleavage selectivity. Cathepsin activity could be measured in individual cells via the use of flow cytometry.

3.1.1.3 Fluorescent molecules for study of *Leishmania* peptidases

Several studies have been undertaken looking at the substrate specificity and activity of *Leishmania* peptidases. For these, fluorescent molecules have been used with both recombinant proteins and cell lysates.

The fluorophore Abz (*ortho*-aminobenzoic acid) has been extensively used for analysis of the activity of recombinant cysteine peptidase B (CPB) isomers. In the first study, a series of fluorogenic substrates related to Abz-KLRFSKQ-EDDnp, (where EDDnp is *N*-[2,4-dinitrophenyl]-ethylenediamine, acting to quench the fluorescence of Abz), were used as a combinatorial fluorescence quenched library to assay the activity of a recombinant isoform of CPB, CPB2.8ΔCTE, (Alves et al., 2001a). The lead substrate, Abz-KLRFSKQ-EDDnp, was cleaved at the R-F bond, so alteration of the P_1 amino acid was investigated. Peptides with charged side chains were hydrolysed and the basic amino acids had the highest catalytic efficiency. The P_3, P_2, and P'_1-P'_3 specificity were also then determined.

In the second study, a series of tetrapeptides were linked to both Abz and K* ([2,4-dinitrophenyl]-ε-NH_2 lysine, which quenches the fluorescence of the Abz). An arginine residue was held constant in the antepenultimate position of the peptide, but the other amino acids were varied. The substrate preference of CPB2.8ΔCTE was compared to that of cathepsin L (Judice et al., 2004). Both enzymes were found to have carboxydipeptidase activity, cleaving the substrates after the arginine residue, with CPB2.8ΔCTE very similar in its specificity to cathepsin L. The peptide FRAK* was the best substrate, in both its free C-terminal carboxyl group form (Abz-FRAK*-OH) and its amidate form (Abz-FRAK*-NH_2), with higher substrate affinity found using the amidate form.

In the third study, the activity of recombinant cysteine peptidase B isoforms, CPB2.8ΔCTE and rCPB3, were compared. CPB3 differs from CPB2.8 in only 3 amino acids and from another isoform, CPB18, in only two, but these changes lead to different substrate preferences (Mottram et al., 1997; Juliano et al., 2004). The amino acids in CPB3 were altered to match those in both CPB2.8 and CPB18 and the substrate specificity of the mutant recombinant proteins was compared to that of the wild-type enzymes, using fluorescent substrates based on Abz-KLRFSKQ-EDDnp (Juliano et al., 2004). The limited amino acid changes led to modifications in the activity of substrate hydrolysis and the S_1 binding site was influenced by the changes between CPB2.8 and CPB3 particularly. In this third study, a 7-amino-4-methylcoumarin (AMC) peptide, Suc-LY-AMC, was also used to determine the activity of the mutated recombinant CPB3 proteins in SDS-PAGE gels (Juliano et al., 2004). This activity is missing from CPB3, but has previously been shown to be present in both CPB2.8 and CPB18, using the substrates Suc-FVR-AMC and Suc-LY-AMC (Mottram et al., 1997).

4-methylcoumarin-7-amide (MCA)-based peptides have been used to investigate the S_2 subsite specificity of recombinant CPB2.8ΔCTE of *L. mexicana*. A variety of substrates based on Bz-FR-MCA were compared (Alves et al., 2001b). A benzoyl (Bz) blocking group was found to give a better substrate with a 10-fold higher rate of degradation than the commercially available benzoyloxycarbonyl (Z) group-based substrate. The fluorophores were used to determine that CPB2.8ΔCTE has a particularly restrictive subsite, more so than cathepsin L.

AMC based substrates have been used to investigate the activity of other peptidases. Firstly, the activity of the *L. major* cathepsin B-like (LmajcatB-like) cysteine peptidase has been investigated with Z-RR-AMC, a cathepsin B substrate, and Z-FR-AMC, a cathepsin B and cathepsin L substrate. LmajcatB-like does not hydrolyse Z-RR-AMC readily, but does hydrolyse Z-FR-AMC, which was postulated to be due to the alteration of a Glu residue in mammalian cathepsin B to a Gly in *L. major* (Chan et al., 1999). This was confirmed by generating a mutated protein with the Glu residue of mammalian enzymes which easily hydrolysed Z-RR-AMC, whilst continuing to hydrolyse Z-FR-AMC.

AMC-containing substrates have also been used to investigate the substrate specificity of recombinant leucyl aminopeptidases (Lap) of *L. amazonensis*, *L. donovani* and *L. major*, using a spectrofluorometer (Morty and Morehead,

2002). L-Leu-AMC was determined to be the best substrate for all three *Leishmania* Lap enzymes. Both metal ion chelators and anti-Lap IgG were found to inhibit the activity of the Lap peptidases against L-Leu-AMC.

Two AMC-linked peptides, Suc-LLVY-AMC and Boc-LRR-AMC, have also been used to test the activity of the purified *L. chagasi* proteasome (Silva-Jardim et al., 2004). The proteasome exhibited higher trypsin-like activity (hydrolysis of Boc-LRR-AMC), than chymotrypsin activity (hydrolysis of Suc-LLVY-AMC), with the proteasome inhibitor lactacystin successfully inhibiting both activities.

Finally, AMC-linked peptides have been used to investigate the *L. major* metacaspase gene expressed in *S. cerevisiae* cells lacking the yeast metacaspase gene. The activity was measured using cell lysates of cells expressing either the full length metacaspase gene or expressing only the catalytic domain or with purified recombinant protein (Gonzalez et al., 2007). Caspase substrates were initially tested, but no significant cleavage of these substrates was detectable, so a number of potential metacaspase substrates were studied, Boc-GRR-AMC, Z-RR-AMC, Z-R-AMC, Z-GGR-AMC and Boc-VLK-AMC. The Z-GGR-AMC substrate was especially highly hydrolysed by the lysate of the catalytic domain-expressing cells, and the activity could be removed by mutating the catalytic dyad amino acids in both the full length and the catalytic-domain proteins.

Bz-RGFFL-4-methyl-β-naphthylamine has been used to investigate aspartyl peptidase activity in *L. mexicana*, where 4-methyl-β-naphthylamine is the fluorescent moiety. The soluble and particulate fractions of lysed promastigotes were assayed for activity (Valdivieso et al., 2007). Peptidase inhibitors were successfully used to determine that the hydrolysis was due to an aspartic peptidase present in the soluble fraction.

3.1.2 AMC-peptides for the investigation of peptidases in live *Leishmania*

On the basis of the preceding studies, it was decided to attempt to analyse peptidase activity using fluorescent peptides, since, although they have not been used for investigation of peptidase activity in live *Leishmania*, their use has been established in a variety of mammalian cells. Recombinant *Leishmania* peptidases and *Leishmania* lysates have been successfully analysed with fluorescent

peptides. It was hoped it would be possible to identify a single peptidase activity in *Leishmania* promastigotes if a small peptide molecule, linked to a fluorophore, were used due to their being fewer possible cleavage sites in a peptide compared to a protein.

Since AMC-containing peptidases have been used for both mammalian live cell assays and *Leishmania* peptidase analysis, peptides using this fluorophore were chosen for the analysis.

3.2 Results

3.2.1 Cleavage of AMC-linked peptide

3.2.1.1 Determination of a suitable AMC-linked peptide for use

The ability of live *Leishmania* to degrade fluorescent peptides was investigated. *L. mexicana* were able to cleave Bz-R-AMC, but unable to cleave Suc-AAPV-AMC or Suc-AAPF-AMC (Figure 3.1). The rate was dependent on the number of promastigotes present. The plateau in the cleavage rate of Bz-R-AMC after approximately 40 minutes was due to the fluorescence reaching the saturation point of the plate reader. The starting fluorescence levels for the three AMC-linked peptides are different as the three have slightly different inherent levels of baseline fluorescence prior to cleavage.

3.2.1.2 Cleavage of Bz-R-AMC by wild-type and mutant parasites

The cleavage of Bz-R-AMC by several *Leishmania* lines was examined. Both *L. mexicana* and *L. major* wild-type (WT) promastigotes were used, as well as *L. mexicana* mutants lacking cysteine peptidases (CPs, Δ*cpb*, Δ*cpa*/Δ*cpb* and Δ*cpc* cells) and *L. major* over-expressing VPS4^{E235Q}, which causes a defect in transport to the lysosome (Besteiro et al., 2006). Both log phase cells and stationary phase cells were evaluated to determine if the developmental stage of the parasites determined the mean rate of cleavage. Deletion of the CPs was found to have no effect on the hydrolysis of Bz-R-AMC and it was also found that lysosomal trafficking defects were only potentially important in stationary phase promastigotes, with a p value of 0.01 for the comparison between *L. major* WT and VPS4^{E235Q} mutant (Figure 3.2).

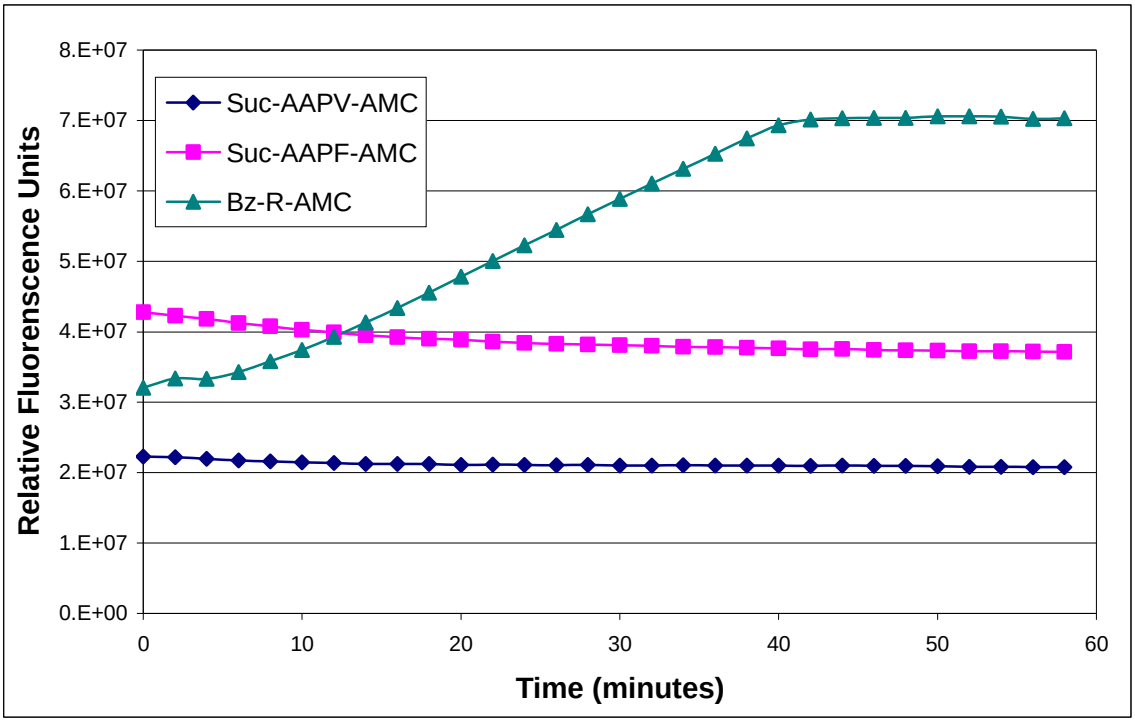


Figure 3.1 Comparison of cleavage of AMC from three peptide-AMC molecules by *L. mexicana* promastigotes. 1 x 10^6 cells were used with 5 µM peptide-AMC.

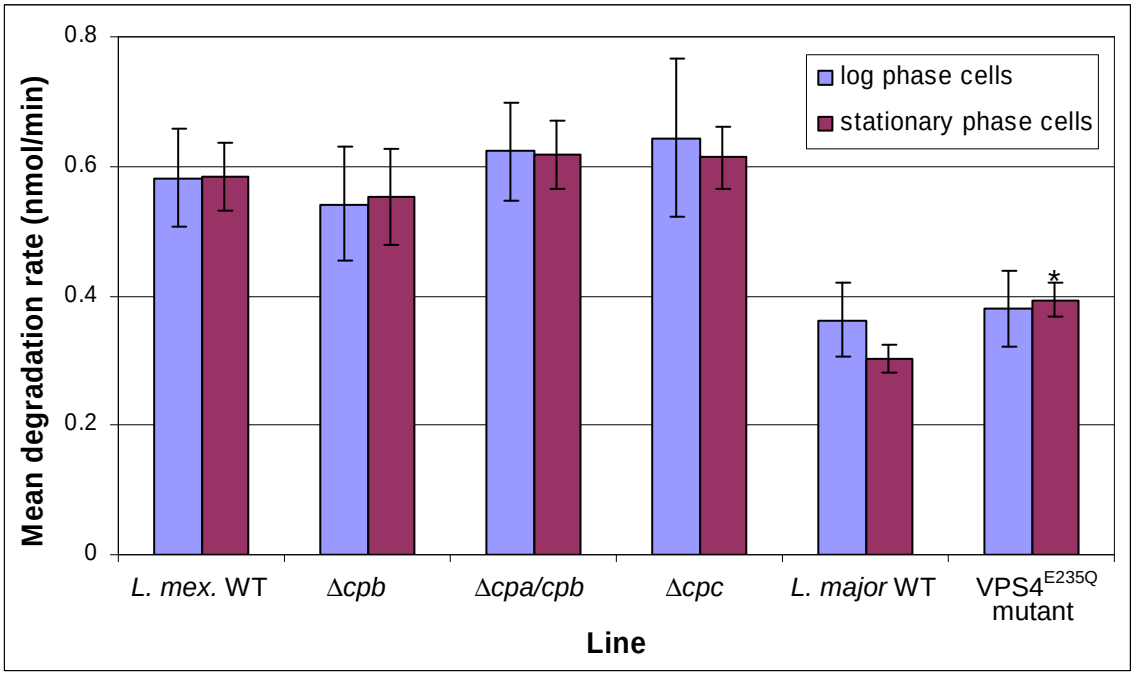


Figure 3.2 Effect of deletion of CP genes or over-expression of a mutated VPS4 on the cleavage rate of 5 µM Bz-R-AMC by *Leishmania* promastigotes. Values are the mean of between 11 and 50 experiments ± SEM. Each experiment was run using 6 x 10^5 cells.
* = p = 0.01 compared to *L. major* WT stationary phase promastigotes, by an unpaired t-test.

3.2.2 Inhibition of degradation using peptidase inhibitors

No difference in the cleavage rate of Bz-R-AMC between WT and mutant *Leishmania* was seen. Peptidase inhibitors were used in an attempt to determine the enzyme responsible for Bz-R-AMC hydrolysis. The following peptidase inhibitors were tested: E-64d, K11777, 1,10 phenanthroline, EDTA, pepstatin A, PMSF, AEBSF, antipain and leupeptin. The structures of the inhibitors are shown in Table 3.1 and the concentrations used are given in Section 2.4.2.

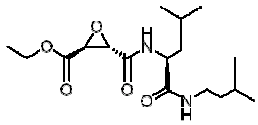
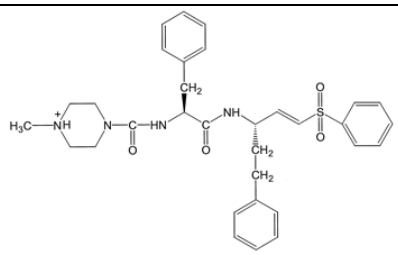
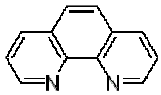
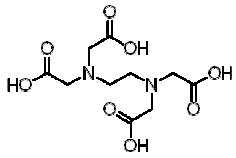
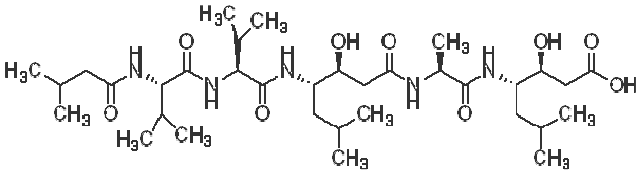
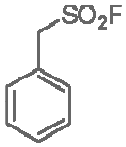
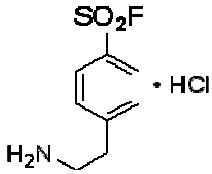
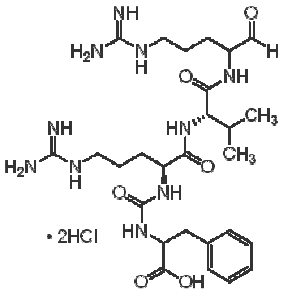
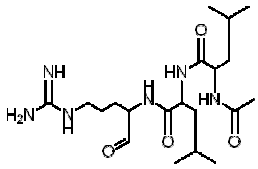
Only antipain, leupeptin and AEBSF were effective inhibitors of Bz-R-AMC degradation (Figure 3.3). This was true for both *L. major* (as shown) and *L. mexicana*.

The IC_{50} values for inhibition of Bz-R-AMC hydrolysis were determined for the three effective inhibitors. Antipain and leupeptin were used to inhibit hydrolysis in log and stationary phase promastigotes of *L. major* and *L. mexicana* WT, *L. mexicana* CP mutants and *L. major* VPS4^{E235Q} mutant cells. AEBSF was only used to inhibit *L. major* stationary phase promastigotes.

The IC_{50} value for antipain was lower for the *L. mexicana* lines than the *L. major* lines, for both log and stationary phase cells. The mutations in the lines had little effect on the IC_{50} values (Figure 3.4). In contrast, the IC_{50} value for leupeptin was lower for the *L. major* lines than the *L. mexicana* lines, for both log and stationary phase cells. As with antipain, any mutation had little effect on the IC_{50} values (Figure 3.4).

AEBSF had a higher IC_{50} than antipain and leupeptin, being 14.4 µM (± a SEM of 0.6), rather than in the nM range as with the former inhibitors.

Table 3.1 Structures of inhibitors used to inhibit degradation of Bz-R-AMC.

Inhibitor	Structure
E-64d	
K11777	
1,10 Phenanthroline	
EDTA	
Pepstatin A	
PMSF	
AEBSF	
Antipain	
Leupeptin	

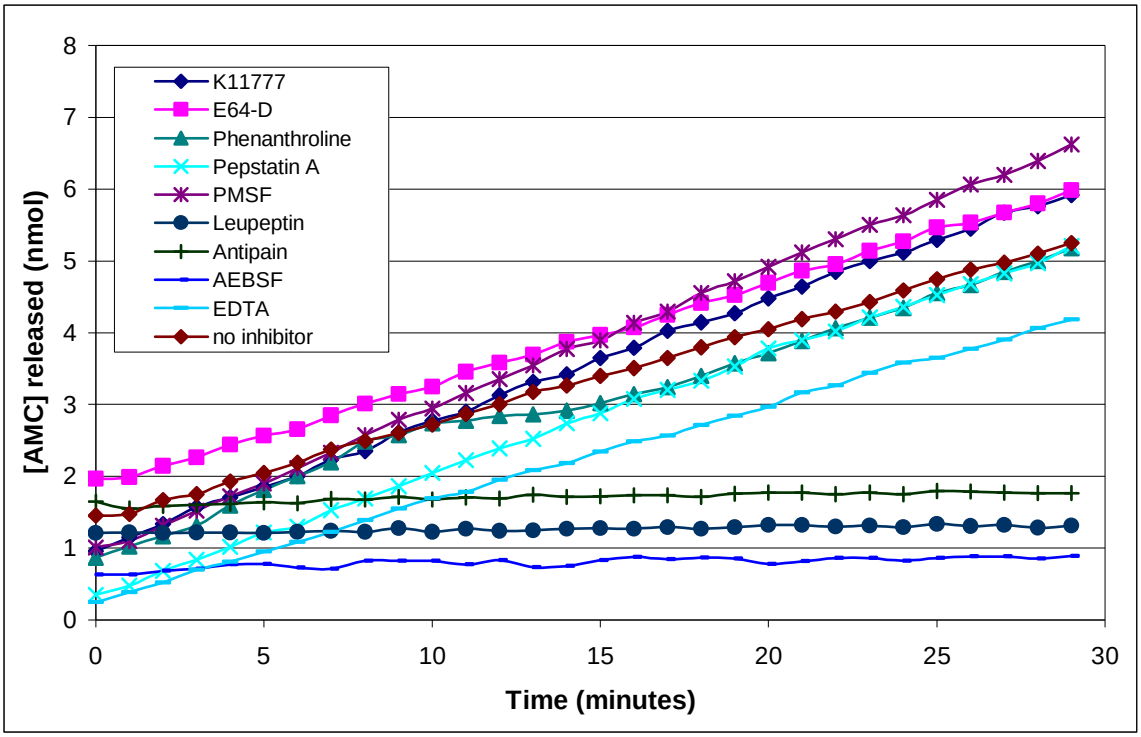
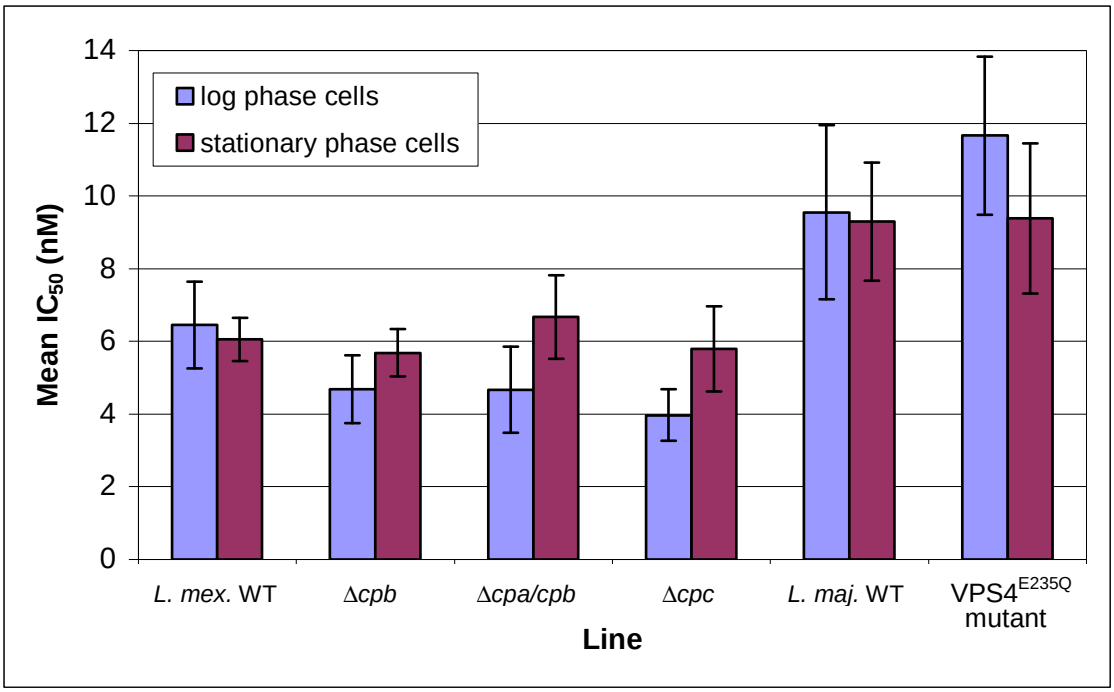


Figure 3.3 Effect of peptidase inhibitors on the cleavage of 5 µM Bz-R-AMC. Peptidase inhibitors were used at the concentrations stated in Section 2.4.2. 6 x 10^5 *L. major* WT stationary phase promastigotes were used for each experiment. A representative graph of at least 3 experiments is shown.

A



B

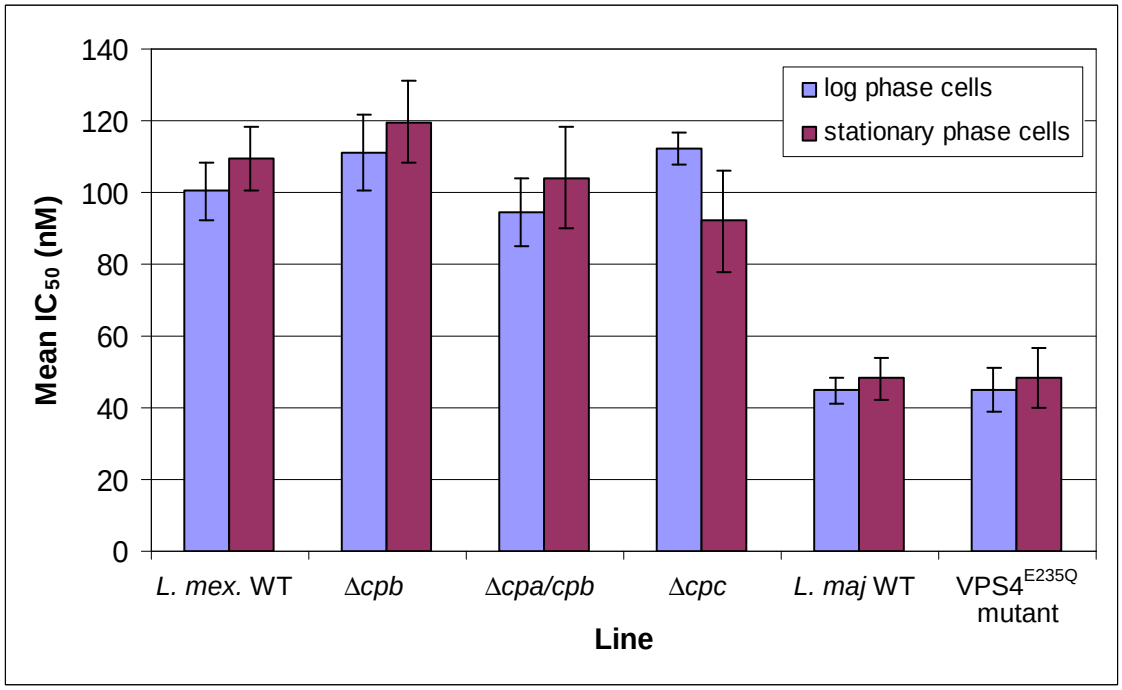


Figure 3.4 Mean IC_{50} values for inhibition of cleavage of 5 µM Bz-R-AMC by antipain and leupeptin. A = inhibition by antipain, B = inhibition by leupeptin. 30 minute assays were performed per experiment. A mean of 5-10 experiments ± SEM is shown. For each experiment 6 x 10^5 *Leishmania* promastigotes were used.

3.2.3 Analysis of Bz-R-AMC uptake

Having determined the inhibitory profile of Bz-R-AMC cleavage, the mechanism of uptake of the peptide was studied. The presence of a transporter was investigated by competition assays. A role for endocytosis was studied via actin inhibition.

3.2.3.1 Arginine transporter

A high affinity arginine transporter has been identified in *Leishmania* (Shaked-Mishan et al., 2006). Since Bz-R-AMC could potentially be taken up through this transporter, an attempt was made to block the uptake of Bz-R-AMC using increasing concentrations of both arginine and pentamidine. The pentamidine was included to preclude the possibility that the arginine was interfering directly with the enzymatic degradation by binding the enzyme active site. Pentamidine was thought to be a substrate of the transporter, but to be unlikely to inhibit the enzyme, following preliminary work in the Mottram laboratory.

Arginine only had an effect at very high concentrations, having an IC_{50} value of 29.7 mM (± a SEM of 4.0).

Pentamidine had a much lower IC_{50} value for blocking the degradation of Bz-R-AMC by live cells, only 47.1 µM (± 16.3). To determine if pentamidine was acting on the peptidase itself, sonicated *L. major* cells were used to cleave Bz-R-AMC, with the lysis removing any role for a transporter in the analysis.

As similar IC_{50} values, given the standard errors, were found for both live cells (47.1 ± 16.3 µM) and sonicated cells (70.7 ± 5.5 µM), it suggests that the pentamidine had an effect on the enzyme itself in both cases and was not affecting the uptake in live cells. The higher IC_{50} in the sonicated cells may be due to other enzymes present in the sonicate which are precluded from hydrolysing Bz-R-AMC normally due to its subcellular location.

3.2.3.2 Endocytosis

To determine whether endocytosis had a role in the uptake of Bz-R-AMC, the inhibitor cytochalasin D was used. This inhibits endocytosis by blocking actin polymerisation via the disruption of actin microfilaments. High concentrations of

cytochalasin D had no significant effect on the cleavage of Bz-R-AMC, with no dose response (Figure 3.5). This suggests that endocytosis is not the primary method by which AMC enters the cell.

To ensure that the concentration of cytochalasin D used was blocking endocytosis, microscopic analysis was carried out. *L. major* stationary phase cells were incubated with either cytochalasin D or DMSO as a control, then FM4-64 was added to both, which is known to be taken up via endocytosis (Besteiro et al., 2006). It was seen that in the presence of cytochalasin D no endocytosis of FM4-64 occurred (Figure 3.6).

3.2.4 Identification of candidate enzymes

The profile of inhibition found suggested that the enzyme(s) potentially responsible for the cleavage of Bz-R-AMC was a serine peptidase. Therefore, an analysis was carried out on the serine peptidases identified by the *L. major* genome project (Ivens et al., 2005). The known inhibitors and the preferred P_1 position amino acids of all the peptidases were compared using the MEROPS Database (http://merops.sanger.ac.uk; Rawlings et al. 2006) and also compared with the profile of Bz-R-AMC hydrolysis.

The results of this analysis (Table 3.2) suggest that oligopeptidase B (OPB, LmjF09.0770) and the other two related oligopeptidase family members (LmjF06.0340 and LmjF35.4020) are likely to be the only enzymes to cleave Bz-R-AMC. The only other possibility is the 26S protease regulatory subunit (LmjF03.0540), since, though these enzymes tend to show preference for larger, polyubiquitinated substrates, they do cleave with a trypsin-like specificity. To test whether the 26S protease was involved, an attempt was made to block cleavage of Bz-R-AMC with the proteasome inhibitors MG132 and lactacystin (Silva-Jardim et al., 2004).

There was no dose response effect of the proteasome specific inhibitors, MG132 and lactacystin, on the hydrolysis of Bz-R-AMC, suggesting that the 26S protease is not responsible for the cleavage (Figure 3.7).

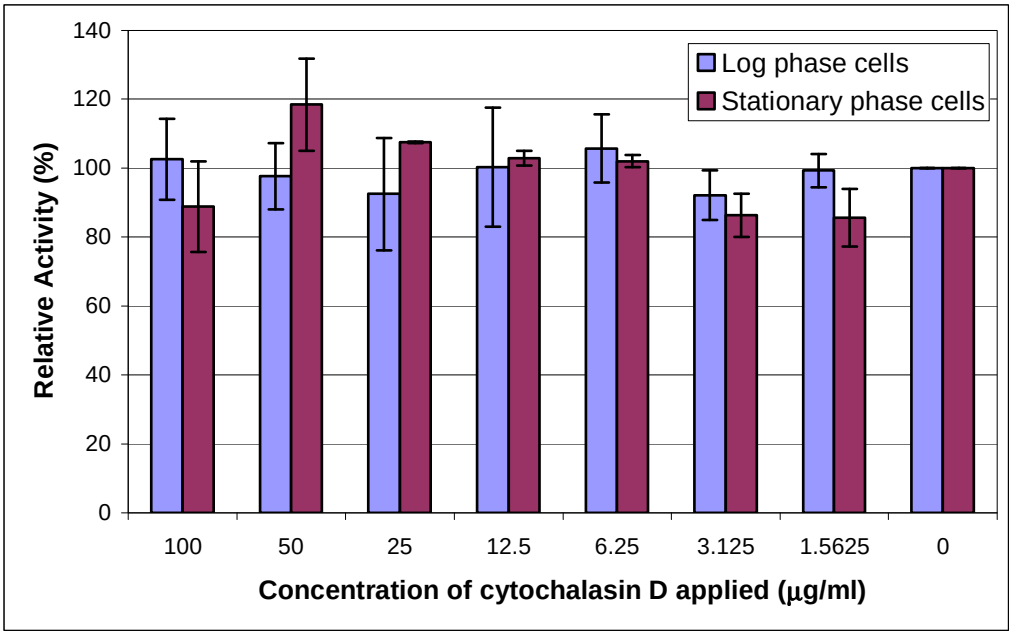


Figure 3.5 Effect of cytochalasin D on cleavage of 5 µM Bz-R-AMC. 3 experiments were completed for both stationary and log phase *L. major* promastigotes, with the mean ± SEM shown. The activity was calculated relative to the uninhibited cells in each particular experiment. 6 x 10^5 cells were used per experiment.

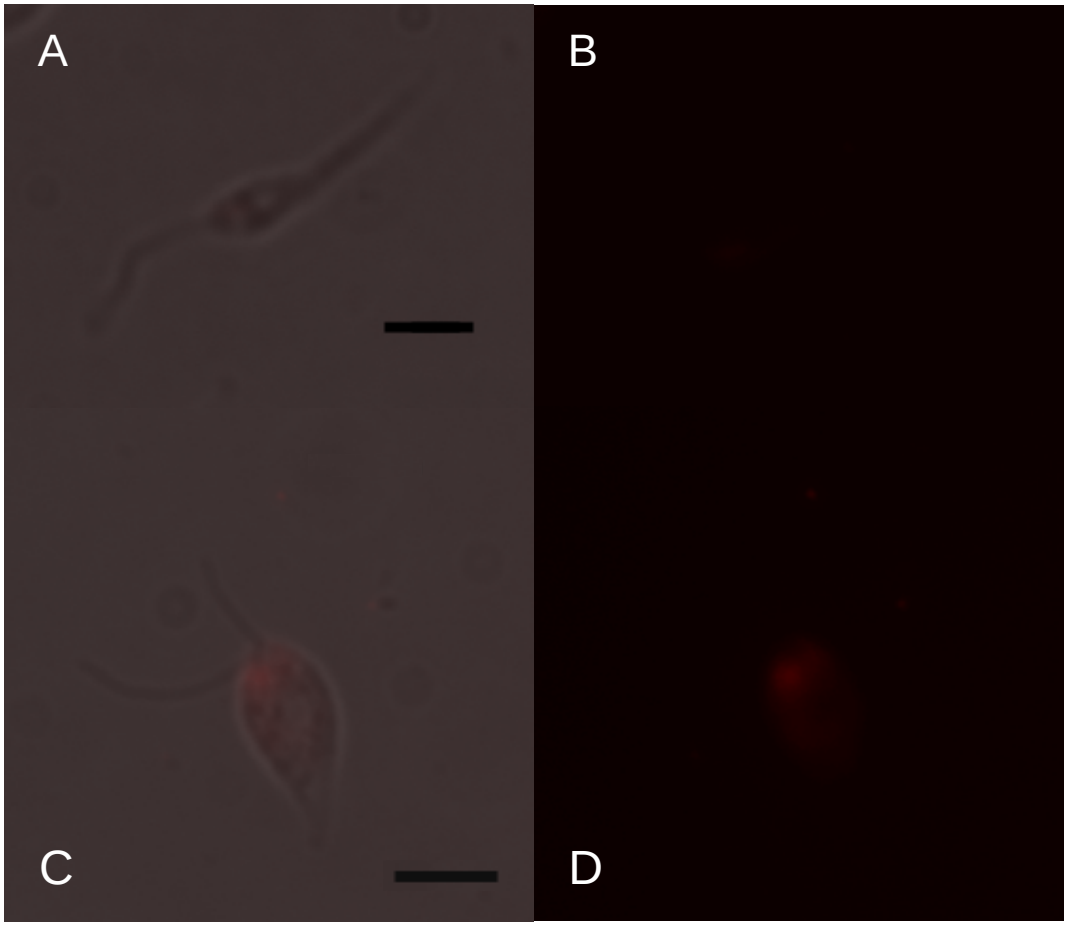
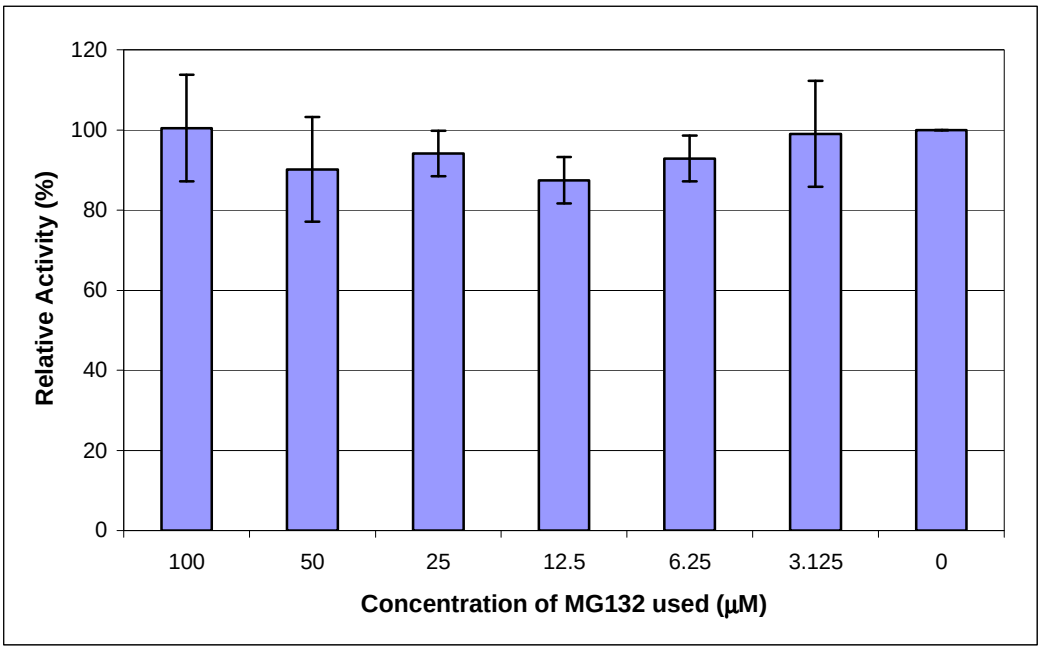


Figure 3.6 Uptake of FM4-64 by *L. major* WT promastigotes. A & B treated with 100 µg/ml cytochalasin D; C & D untreated. A & C = merge of phase and FM4-64, B & D = FM4-64 alone. Scale = 5 µm.

Table 3.2 Serine peptidases found in *L. major* genome and their likelihood for being the enzyme responsible for the cleavage of Bz-R-AMC.

Gene	Description	Clan	Family	Cleavage likely?	Reason
LmjF02.0430	Rhomboid-like	S-	S54	X	Prefer membrane bound, larger substrates.
LmjF04.0850	Rhomboid-like	S-	S54	X	
LmjF13.1040	Subtilisin-like serine peptidase	SB	S8-like	X	Inhibited by PMSF
LmjF28.2380	Subtilisin-like serine peptidase	SB	S8-like	X	
LmjF12.1330	Serine peptidase, putative	SC	S9D	X	Homologues have preference for cleaving after proline
LmjF36.2420	Dipeptidyl-peptidase 8-like serine peptidase, putative	SC	S9B	X	Related peptidases prefer cleaving after proline, or less well alanine or hydroxyproline
LmjF36.6750	Prolyl oligopeptidase, putative	SC	S9A	X	Cleaves after proline, not arginine
LmjF09.0770	Oligopeptidase B	SC	S9A-like	Possible	Post arginine cleavage, homologues in *T. brucei* inhibited by antipain and leupeptin
LmjF06.0340	Oligopeptidase B-like protein	SC	S9A-like	Possible	Closely related to LmjF09.0770
LmjF35.4020	Bem46-like serine peptidase	SC	S9X	Possible	Closely related to LmjF09.0770
LmjF02.0710	ATP-dependent Clp protease subunit, HSP78	Putative		X	Clp inhibited by EDTA
LmjF27.2630	ATP-dependent Clp protease subunit, HSP78	Putative		X	
LmjF29.1270	ATP-dependent Clp protease subunit, HSP100	Putative		X	
LmjF30.1700	ATP-dependent Clp protease subunit, heat shock protein	Putative		X	
LmjF33.0400	ATP-dependent Clp protease subunit, heat shock protein	SC	S9D	X	
LmjF18.0450	Serine carboxypeptidase (CBP1), putative	SC	S10	X	Preference for cleaving after hydrophobic groups
LmjF28.1950	X-pro, dipeptidyl-peptidase, serine peptidase, putative	SC	S15	X	Inhibited by PMSF and post proline-cleaving
LmjF08.0450	Signal peptidase type I, putative	SF	S26A	X	Prefers small residues at P1
LmjF36.0200	Mitochondrial inner membrane signal peptidase, putative	SF	S26B	X	Stimulated by metal ion chelators
LmjF27.0380	Nucleosporin, putative	SP	S59	X	Cleaves after phenylalanine, not arginine
LmjF03.0540	26S protease regulatory subunit, putative	SJ	S16	Unlikely	26S protease preferentially cleaves longer peptide chains covalently conjugated to polyubiquitin chains, but can cleave with trypsin-like specificity (depends on species)

A



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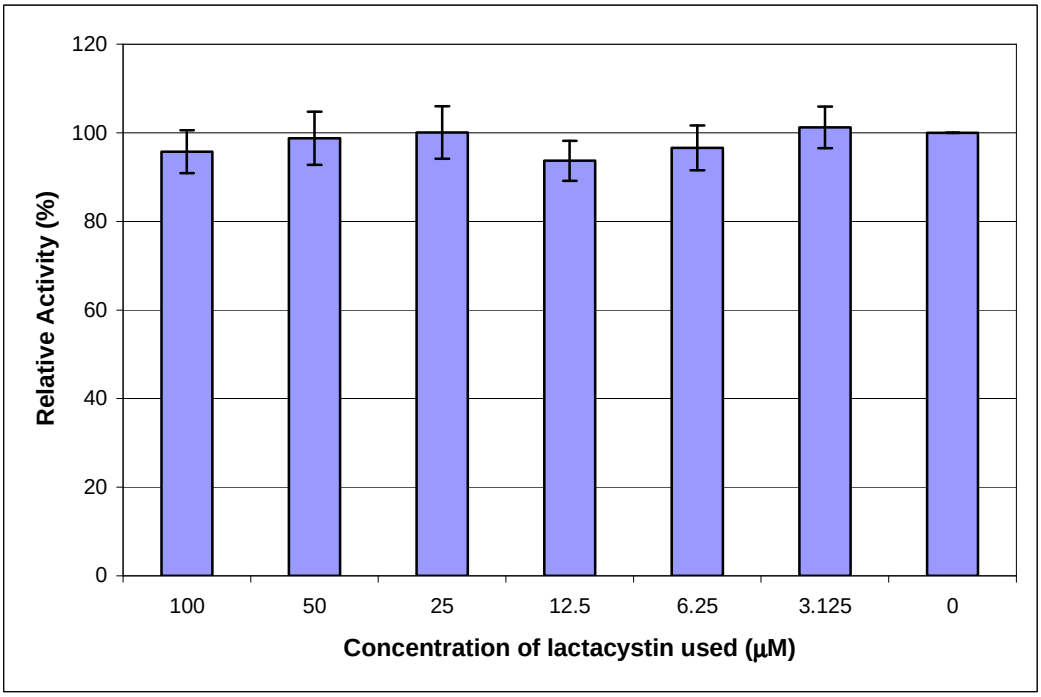


Figure 3.7 Effect of MG132 and lactacystin on the cleavage of 5 µM Bz-R-AMC by *L. major* WT stationary promastigotes. Relative activities compared to no inhibitor are shown. A = MG132, B = lactacystin. Mean of four experiments ± SEM. The activity was calculated relative to the uninhibited cells in each particular experiment. 6 x 10^5 cells were used per experiment.

3.3 Discussion

Leishmania peptidases have been identified as being important virulence factors and as such peptidase activity was investigated in live cells.

Bz-R-AMC was the only peptide tested that was successfully hydrolysed by *Leishmania* promastigotes (Figure 3.1). The rate of hydrolysis of Bz-R-AMC by both *L. mexicana* and *L. major* wild-type promastigotes were determined and compared to mutant lines previously described (Mottram et al., 1996b; Bart et al., 1997; Besteiro et al., 2006), to investigate whether any of the three main clan CA cysteine peptidases present in *Leishmania* were responsible for the cleavage of Bz-R-AMC or if mis-trafficking to the lysosome would interfere with the hydrolysis. There was a significant difference between the two species, with *L. mexicana* having higher activity compared to *L. major* in both log and stationary phase promastigotes (with p values of 0.03 and 0.00001 respectively, by unpaired t-test), but there was no difference between the log and stationary phase cells for all lines (Figure 3.2). This difference between the two species is interesting; there are several possible reasons. There could be a difference in either the uptake or degradation of the substrate, as both are being measured at once. Potentially, there could be a difference in the membrane properties between the species that affects uptake. If the difference is due to the degradative properties of the species it could be that the peptidase(s) responsible may be more highly expressed in *L. mexicana*, or different proportions of peptidases capable of cleaving Bz-R-AMC (as identified in Table 3.2), could be expressed in the two species.

Deletion of cysteine peptidases did not result in an alteration of the hydrolysis and, as such, these peptidases cannot be responsible for the cleavage. On the other hand, the VPS4^{E235Q} mutant stationary phase cells had a significantly higher rate of cleavage than the *L. major* WT stationary phase cells, with a p value of 0.01 (Figure 3.2). However, VPS4^{E235Q} mutant promastigotes do not differentiate to metacyclic promastigotes in culture (Besteiro et al., 2006) and so a stationary phase culture of this line is not comparable to a metacyclic-containing, stationary phase culture of the WT line. It is likely that the difference found in the stationary cultures is due to the differences in the cells that compose the culture, especially given that the VPS4^{E235Q} mutant was not different to the WT line in the log phase

of culture, with a p value of 0.82. This finding suggests that the peptidase cleaving Bz-R-AMC is not present in the lysosomal system.

Since the major cysteine peptidases were not hydrolysing Bz-R-AMC, a series of general peptidase inhibitors were used to investigate the type of peptidase that was involved. The activity was determined to be due to a peptidase susceptible to antipain, leupeptin and AEBSF (Figure 3.3). Antipain and leupeptin affect both cysteine peptidases and serine peptidases which have a trypsin-like specificity for basic residues in the P_1 site. Specific cysteine peptidase inhibitors were ineffective, whereas, the serine peptidase inhibitor AEBSF did inhibit this activity, although with a high IC_{50}. These data indicate that the peptidase involved is a serine peptidase with trypsin-like specificity.

The IC_{50} values for antipain and leupeptin were determined for *L. mexicana* and *L. major* WT, CP mutants and the $VPS4^{E235Q}$ mutant, and neither the mutations nor the cell growth phase had an effect on the IC_{50} values (Figure 3.4). The IC_{50} values were different between the two species however, with antipain having a lower IC_{50} for *L. mexicana* and leupeptin a lower IC_{50} with *L. major*. Leupeptin has a modified tripeptide structure, whereas antipain contains a tripeptide linked to a large [carboxy-phenylethyl]-carbamoyl structure (Table 3.1). This suggests that the difference may be due to the size of the inhibitor, and perhaps, as with the difference in rate of cleavage between the two species, it is due to a difference in ease of uptake of the inhibitor through the membrane in each species. Alternatively, more than one peptidase could be involved, each of which is affected by leupeptin and antipain to different degrees and which could be expressed at different levels in the two species.

The IC_{50} value for AEBSF inhibition of the cleavage in *L. major* stationary promastigotes was found to be higher than that of antipain or leupeptin, being in the low µM range rather than nM. AEBSF is a very similar inhibitor to PMSF (Barrett and Rawlings, 1995), an inhibitor that had no effect on the cleavage (Figure 3.3). It is likely that the differences in their activity are due to the small differences in their structures, viz. PMSF having an extra methyl group between the benzyl ring and the sulfonylfluoride compared to AEBSF and AEBSF having an additional aminoethyl group attached to the benzyl ring, which PMSF lacks (Table 3.1). These differences are likely to determine that AEBSF, but not PMSF, can interact with the active site of the peptidase and inhibit it.

The method by which Bz-R-AMC entered *Leishmania* was also investigated. An arginine transporter, LdAAP3, which mediated high affinity transport of arginine across membrane was described in *L. donovani* in 2006 (Shaked-Mishan et al., 2006). This transport was inhibited by the anti-*Leishmania* drug pentamidine, which is a diamidine. It was hypothesised that it could be the means by which Bz-R-AMC was entering the cell. However, arginine was not found to be a very good inhibitor of hydrolysis, only affecting cleavage with an IC_{50} of 29.7 mM. LdAAP3 was inhibited over 80% by arginine at a concentration of 100 µM (Shaked-Mishan et al., 2006), suggesting that it is very unlikely that the transporter is responsible for uptake of Bz-R-AMC. The IC_{50} value is therefore likely to be of arginine inhibiting the peptidase itself. As it is such a high value, this suggests that the active site pocket of the peptidase is not simply binding the arginine residue after which it cleaves; interactions between the S_2 or S_3 sites, or more, and the ligand are also important.

Pentamidine was also used to inhibit the cleavage of Bz-R-AMC. The IC_{50} value for pentamidine was far lower than that of arginine, suggesting it was also affecting the peptidase itself. Pentamidine was used to inhibit cells lysed by sonication and was found to inhibit the cleavage of Bz-R-AMC. Pentamidine was therefore inhibiting the peptidase rather than inhibiting uptake of the substrate, further corroborating that the arginine transporter is not the mode of uptake for Bz-R-AMC. Pentamidine is also a larger molecule than arginine and the lowered IC_{50} value of pentamidine compared to arginine suggests that it fits into the active site pocket and interacts more suitably, assuming that it is inhibiting the activity competitively.

In addition, the potential for endocytosis to be the route of uptake of Bz-R-AMC was investigated, using cytochalasin D. This inhibitor is commonly used to prevent endocytosis but was found to be ineffective at interfering with hydrolysis of Bz-R-AMC, implying that the lack of endocytosis was not preventing uptake of the substrate (Figure 3.5). Cytochalasin D inhibition of endocytosis was confirmed using the lipophilic dye FM4-64, which previously has been shown to be taken up through the flagellar pocket and trafficked through the endosomal system to the lysosome (Besteiro et al., 2006). It was confirmed that FM4-64 entered the cell when uninhibited, but the uptake was prevented when cytochalasin D was applied (Figure 3.6).

Bz-R-AMC has been found not to enter through the arginine transporter nor via endocytosis, although it is inevitable that some would be taken up by endocytosis when present in the extracellular milieu. It seems likely that the Bz-R-AMC is entering *Leishmania* by simple diffusion through the cell membrane. It is a small molecule, which is likely to cross membranes, especially as its stock is dissolved in dimethylformamide, which aids entry of even lipophilic substrates into cells (Boonacker and Van Noorden, 2001). However the involvement of a different transporter has not been formally ruled out.

The mode of entry suggests that the peptidase responsible for the cleavage is unlikely to be located within the endosomal system or lysosome, which would correlate with the unimpaired degradation found with the VPS4 mutant line, which has a defect in transport to the lysosome and thus alterations in the peptidases found in that compartment. The peptidase involved is likely to be cytosolic.

An analysis of the serine peptidases of *L. major* (Ivens et al., 2005), together with the profile of inhibition and the fact that the enzyme cleaves post arginine, led to the conclusion that OPB and its related family members were the peptidases most likely to be responsible for the activity observed (Table 3.2). The possibility that the 26S protease could be involved was excluded by the use of the specific inhibitors MG132 and lactacystin (Figure 3.7).

3.3.1 Conclusions

Leishmania promastigotes provide an excellent system for the characterisation of peptidases in living cells. Bz-R-AMC was found to be a good substrate for hydrolysis by *Leishmania*. It is suggested that the substrate may be entering the cell by diffusion, as it is not entering through the endosomal system nor the arginine transporter.

The inhibitory profile of this activity was determined using standard peptidase and proteasome inhibitors. The inhibition by antipain, leupeptin and AEBSF is indicative of a trypsin-like serine peptidase. Genomic data identified OPB and two other related peptidases as the most likely candidate peptidases and so OPB will be investigated to determine whether the correct identification has been made.

Chapter 4

OLIGOPEPTIDASE B OF *L. MAJOR*

4.1 Introduction

Oligopeptidase B (OPB) is a member of the S9 family of serine peptidases, the prolyl oligopeptidase (POP) family, which is part of clan SC. Oligopeptidase B is restricted taxonomically, being found only in bacteria, plants and trypanosomatids. All OPBs cleave post-basic residues, preferring arginine, in a trypsin-like activity (Rea and Fulop, 2006).

4.1.1 Peptidases of the POP family

The POP family contains prolyl oligopeptidase, dipeptidyl peptidase IV, acylaminoacyl peptidase and oligopeptidase B (Polgar, 2002). The POP family have a conserved Ser/Asp/His catalytic triad, found in their carboxy terminal regions, and are restricted to hydrolysing peptides up to around 30 amino acids in length.

4.1.1.1 Structure of POP and OPB

POP has been demonstrated to be monomeric (Fulop et al., 1998). The restriction in the length of substrates cleaved is due to the conserved peptidase structure: the carboxy terminal region of the peptidase is a catalytic domain and the amino terminal region is a seven-bladed β-propeller that restricts access to the active site through the central tunnel (Fulop et al., 1998; Polgar, 2002). The active site of POP is in a cavity in the interface between the two domains, covered by the central tunnel of the β-propeller (Fulop et al., 1998). The β-propeller is not covalently closed and may oscillate between open and closed forms for substrate binding and catalysis (Fulop et al., 2000).

There is only 22% amino acid identity between human POP and *E. coli* OPB (Venalainen et al., 2004), but there is seemingly high structural similarity. A model of *E. coli* OPB was constructed based on porcine POP. The two were very similar, though the binding site of *E. coli* OPB was more negatively charged than that of POP. Two OPB substrates were docked into the active site and it was found that the best substrate, Z-Arg-Arg, was a better fit into a hydrophilic region (Gerczei et al., 2000). *L. amazonensis* OPB has also been modelled based on porcine POP and has a very similar structure, though again with a more negative surface and active site region compared to POP (de Matos Guedes et al., 2007).

L. major OPB has been crystallised by Prof. Neil Isaacs' group at the University of Glasgow and its structure solved. It was found to be very similar in structure to porcine POP, with 86% similarity in the secondary structures. Analysis and comparison of the OPB and POP structures is ongoing. The structures are shown in Figure 4.1.

4.1.2 Bacterial Oligopeptidase B

Oligopeptidase B was identified in *Escherichia coli* as a cytosolic enzyme, originally designated as Protease II (Pacaud and Richaud, 1975). Purified *E. coli* OPB has optimal activity at pH 7.5 and was competitively inhibited by the serine peptidase inhibitors *N*-tosyl-L-lysyl-chloromethylketone (TLCK) and diisopropyl phosphorofluoridate (DFP), but not by phenylmethylsulfonyl fluoride (PMSF). The trypsin inhibitors benzamidine and *p*-aminobenzamidine also inhibited OPB. L-arginine also competitively inhibited activity.

Kanatani et al. sequenced *E. coli OPB* and calculated a predicted molecular weight for the corresponding protein of 81.9 kDa. The expressed recombinant *E. coli* OPB was inhibited by DFP, TLCK and antipain. *E. coli* OPB was found to have 25.3% homology to porcine brain prolyl oligopeptidase, which led to its inclusion in the prolyl oligopeptidase family (Kanatani et al., 1991). It was also found to be inhibited by protamine, a basic peptide containing polyarginine sequences (Tsuji et al., 2006). *E. coli* OPB was subsequently found to have a preference for di-arginine containing compounds over single arginine containing substrates and to cleave peptides up to 30 amino acids in length (Polgar, 1997).

OPBs from *Rhodococcus erythropolis*, *Moraxella lacunata*, *Treponema denticola* and *Salmonella enterica* have also been characterised, and OPB genes are present in the genomes of other bacterial species (Venalainen et al., 2004). *R. erythropolis* OPB was purified and estimated to have a molecular weight of 82 kDa by SDS-PAGE, a similar size to *E. coli* OPB (Shannon et al., 1982). It was inhibited by DFP, TLCK, *p*-aminobenzamidine and antipain, similarly to *E. coli* OPB, and also by leupeptin, ovomucoid and gramicidin S. PMSF was ineffective at inhibiting *R. erythropolis* OPB.

OPB from *M. lacunata* was also originally designated as protease II (Yoshimoto et al., 1995). The gene was expressed in *E. coli* and the nucleotide sequence found,

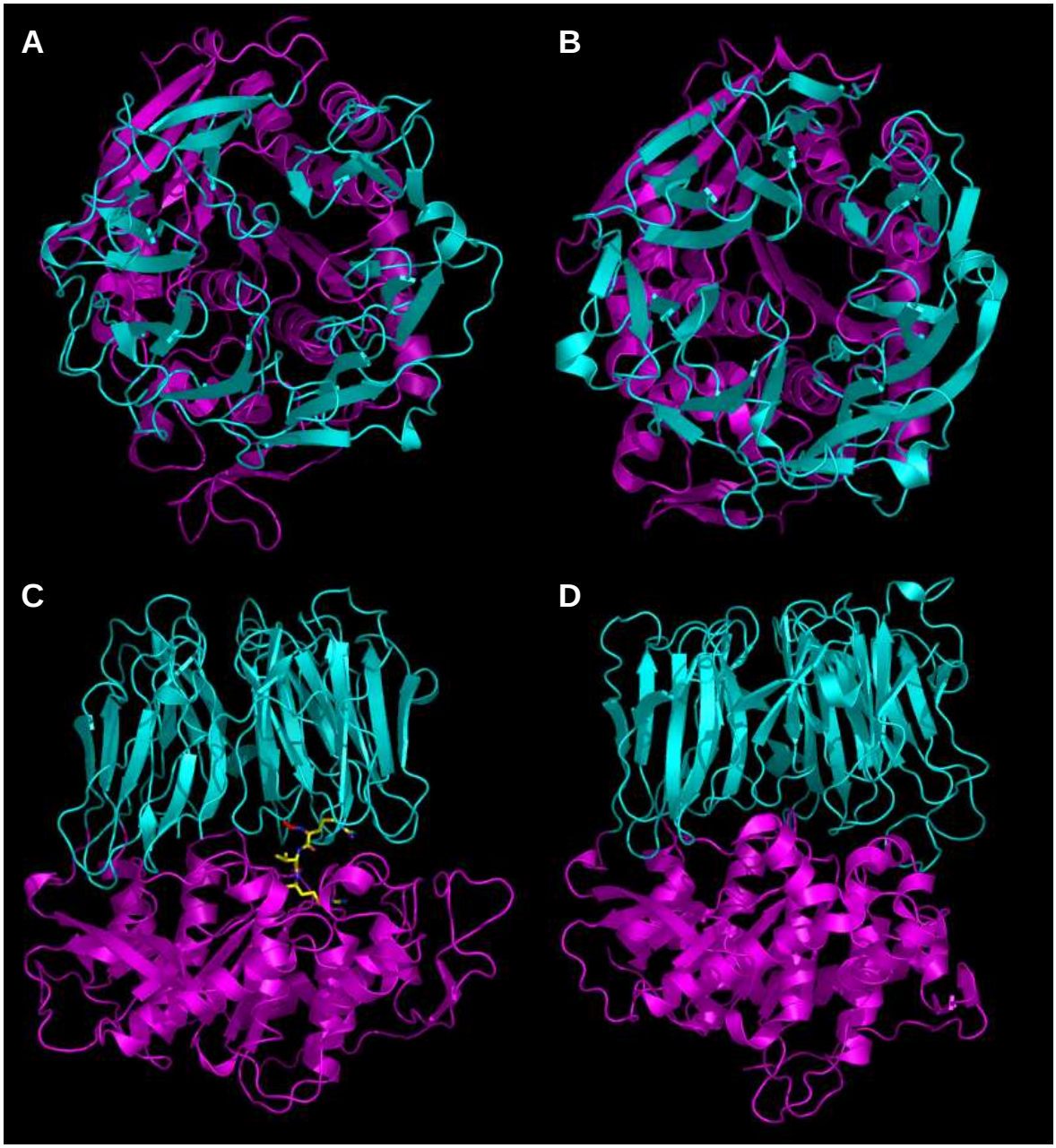


Figure 4.1 Structure of *L. major* OPB and porcine brain POP. A and C = *L. major* OPB, B and D = porcine brain POP. Cyan = β-propeller, magenta = catalytic domain. A and B show the view down the pore of the β-propeller onto the catalytic domain, C and D show a side view. The inhibitor antipain was co-crystallised with OPB and is shown in yellow, blue and red in C, bound into the active site. The diagrams were constructed using PyMol (Delano Scientific) by Dr. Nicholas D. Bland (University of Glasgow), using data provided by Prof. N. Isaacs (University of Glasgow; A and C) and of structure 1h2w from RCSB Protein Data Bank (B and D).

to give a predicted molecular weight for the encoded protein of 79.5 kDa and 38% identity to *E. coli* OPB. Recombinant *M. lacunata* OPB was also inhibited by DFP, TLCK and *p*-chloromercuribenzoic acid (PCMB).

T. denticola OPB was purified, partially sequenced and found to have a molecular weight of 78 kDa by SDS-PAGE. Purified *T. denticola* OPB hydrolysed peptide substrates, but not proteins (Makinen et al., 1995). The gene encoding *T. denticola* OPB was subsequently isolated and was found to have significant homology to *E. coli* OPB and other POP family members (Fenno et al., 2001). An OPB mutant was generated, truncating OPB, resulting in an inability to hydrolyse the OPB substrate *N*-α-benzoyl-L-arginine-*p*-nitroanilide (BA*p*NA), showing OPB was responsible for all the trypsin-like activity in *T. denticola*.

S. enterica OPB has 75% identity to *E. coli* OPB. Recombinant *S. enterica* OPB cleaved a number of 7-amino-4-methylcoumarin (AMC)-linked peptides, with the best substrate being Cbz-RR-AMC (Morty et al., 2002), as with *E. coli* OPB (Polgar, 1997). *S. enterica* OPB was inhibited by the serine peptidase inhibitors PMSF (unlike *E. coli* OPB), 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF or pefabloc), 2,3-dichloroisocoumarin, antipain and leupeptin. Limited post-arginine hydrolysis of histone proteins was found, but only in regions of protein lacking secondary structure.

S. enterica OPB was used to investigate the substrate recognition properties of OPB enzymes (Morty et al., 2002). The amino acids of the catalytic domain of POP and OPB have been compared and nine residues varying between the two were mutated. For P_1 substrate specificity Glu^{576} and Glu^{578} were important, with mutation of either alone to alanine reducing hydrolysis of Cbz-R-AMC and mutation of both (to alanines, arginines or tryptophan and threonine respectively) abolished it completely. For P_2 substrate specificity, the mutations Asp^{460}Thr and Asp^{462}Asn reduced hydrolysis of Cbz-RR-AMC and Cbz-FR-AMC, though the effect was lower with Cbz-FR-AMC, suggesting involvement in the preference for cleavage after two basic amino acid residues. Mutation of both residues had a larger effect on hydrolysis than either single mutation (Morty et al., 2002).

4.1.3 Oligopeptidase B in plants

Trypsin-like peptidases with the characteristics of OPB have been described in plants, in soy (*Glycine max*), ragweed (*Ambrosia artemisiifolia*) and wheat (*Triticum aestivum*), although only the last enzyme has been designated OPB.

Soybean seed trypsin-like peptidase was found to be inhibited by DFP, PCMB, antipain and leupeptin, but not PMSF (Nishikata, 1984). Cultured soybean cells had a trypsin-like peptidase with a molecular weight of 90 kDa (Guo et al., 1998). As with the soybean seed peptidase, it was inhibited by DFP, 3,4-dichloroisocoumarin (DCI), AEBSF, antipain and leupeptin, but not PMSF. Sequenced peptides had homology to both *E. coli* OPB and human and pig POP.

Ragweed trypsin-like peptidase was purified from pollen extract and found to have a molecular weight of 80 kDa by SDS-PAGE (Bagarozzi et al., 1998). It was inhibited by DFP, TLCK, DCI and a number of synthetic serine inhibitors, but not by leupeptin. It preferentially cleaved peptides post Arg-Arg or Gly-Arg residues and was found to cleave a number of biologically active peptides involved in maintaining bronchial function. Ragweed OPB may thus be at least partially responsible for the known role of ragweed pollen in stimulating respiratory symptoms in hay fever and asthma sufferers.

Wheat OPB was inhibited by DFP, TLCK, leupeptin, antipain and benzamidine (Tsuji et al., 2004). OPB activity increased in germinating cells. Wheat OPB peptides were sequenced and found to have high homology to *E. coli* OPB. It was also inhibited by protamine, as with *E. coli* OPB (Tsuji et al., 2006).

4.1.4 Oligopeptidase B in *Trypanosoma cruzi*

T. cruzi OPB (TcOPB) was described in 1997 as a cytosolic serine endopeptidase, present in all life cycle stages, with significant homology to *E. coli* and *M. lacunata* OPBs (Burleigh et al., 1997). Recombinant TcOPB had a molecular weight of 81 kDa by SDS-PAGE, but a weight of ~120 kDa by non-reducing gel electrophoresis. When purified, OPB activity was found in elutions from columns where proteins around 180 kDa were expected, suggesting OPB is dimeric. TcOPB was inhibited in a similar manner to *E. coli* OPB, being inhibited by PCMB, leupeptin, antipain and TLCK, but not PMSF. Activity was also inhibited up to 90%

by anti-recombinant TcOPB antibodies. Longer peptides, with several binding sites in the active site filled, were cleaved most efficiently, with di-basic residues in P_1 and P_2 and hydrogen donors in the P_1' positions preferred (Hemerly et al., 2003). 17-mer peptides could be hydrolysed, but not 21-mer peptides.

TcOPB in trypomastigote soluble extract (TSE) had been previously postulated to induce Ca^{2+} transients in host cells (Burleigh and Andrews, 1995). This was confirmed when anti-OPB antibodies led to significant delay in the induction of the transients. TcOPB does not signal directly for Ca^{2+} release, but is responsible for generating a Ca^{2+}-signalling factor (Burleigh et al., 1997). *T. cruzi* Δ*opb* mutants were generated with a gene dosage effect found; heterozygote extracts had 50% OPB activity and Δ*opb* none (Caler et al., 1998). Heterozygotes were approximately 40% less infective to mammalian host cells than wild type trypomastigotes; Δ*opb* trypomastigotes were 75% less infective. Addition of recombinant OPB to Δ*opb* TSE restored Ca^{2+} signalling ability, whilst addition of an inactive recombinant active site mutant (Ser^{565}Thr) OPB did not. Parasitaemia of infected mice was also reduced when heterozygote or Δ*opb* trypomastigotes were used as the infectants.

The presence of anti-OPB antibodies in infected animals and patients was investigated using purified TcOPB. Approximately 72% of infected animals had antibodies cross-reactive with TcOPB, whilst mock infected animals did not. Similarly, 86% of chagasic patients, 90% of mucocutaneous leishmaniasis patients and 55% of visceral leishmaniasis patients had sera reactive to TcOPB, but control malarial patients did not (Fernandes et al., 2005). Antibodies from chagasic patients did not inhibit the activity of purified TcOPB, with the OPB activity enhanced by human sera, by an unknown mechanism. Non-chagasic patient sera also enhanced OPB activity.

Trypomastigotes, but not epimastigotes, were found to release TcOPB, as OPB activity increased in the culture supernatant over a 1 hour period. The control activity of cathepsin D or cruzipain did not increase over the same time period, indicating no cell lysis occurred (Fernandes et al., 2005). This contrasts with the results of Caler *et al*. who only found TcOPB in the cytosol by EM immuno-localisation, with none in the flagellar pocket, the route of exocytosis from trypanosomes (Caler et al., 1998).

4.1.5 Oligopeptidase B in African trypanosomes

Trypanosoma brucei brucei OPB (TbOPB) was initially characterised as an 80 kDa endopeptidase in 1992. It cleaved post basic residues and was inhibited by DFP. Dithiothreitol (DTT) increased the stability of the peptidase, and the thiol blocking agents iodoacetamide and *N*-ethylmaleidide led to loss of activity, suggesting a thiol group is necessary for activity (Kornblatt et al., 1992). TbOPB was confirmed as a member of the POP family by sequence comparison with *T. cruzi* and bacterial OPBs (Morty et al., 1999b). TbOPB was also recognised by antibodies raised against TcOPB.

Activation of TbOPB by a number of thiol reducing agents, particularly DTT, has been confirmed (Troeberg et al., 1996; Morty et al., 1999b). By mutation of the cysteine residues in TbOPB to serine residues, C^{256} was found to be necessary for inhibition by iodoacetamide and *N*-ethylmaleidide and C^{559} and C^{597}, with possibly C^{256}, were necessary for thiol activation of TbOPB (Morty et al., 2005b).

Further inhibitors of TbOPB were later determined: AEBSF, leupeptin and antipain (Troeberg et al., 1996), DCI and PMSF (Morty et al., 1999b). E64 was also inhibitory, which is unusual for a serine peptidase and has not been reported for non-African trypanosome OPBs (Morty et al., 1999b). Mammalian peptidase inhibitors, including serpins, did not inhibit TbOPB, probably due to their large size. Protein substrates were not cleaved but peptides were, up to a limit of 4 kDa, including a number of peptides known to be present in the host bloodstream (Troeberg et al., 1996). Substrate binding was better with both P_1 and P_2 sites occupied (Morty et al., 1999b) and improved further with multiple substrate binding sites occupied (Hemerly et al., 2003). As with TcOPB, 17-mer peptides could be hydrolysed, but not 21-mer peptides.

The trypanocidal agents pentamidine, diminazene and suramin are inhibitors of TbOPB, as are a number of suramin analogues (Morty et al., 1998). Pentamidine and diminazene contain arginine-like motifs, mimicking OPB substrates, whilst suramin has been shown to inhibit other serine peptidases. TbOPB may be a target of suramin as significant correlation between OPB inhibition and trypanocidal efficacy of the suramin analogues has been found (Morty et al., 1998). Other inhibitors of TbOPB, containing arginine at the P_1 position, have also been found to have antitrypanosomal activity. The best compounds could improve

survival rates of *T. brucei* infected mice, although they were toxic in high doses (Morty et al., 2000).

TbOPB activity was detected in the bloodstream of infected rats, based on the inhibitory profile found (Troeberg et al., 1996), western immunoblotting of rat plasma and the ability of anti-TbOPB antibodies to block most cleavage of Cbz-RR-AMC by the plasma (Morty et al., 2001). TbOPB was purified from rat plasma, identified by sequence analysis and found to be fully active against a range of AMC-linked peptides. TbOPB was not released into the culture supernatant, indicating that cell lysis is responsible for its presence in plasma.

OPBs have also been purified from *T. congolense* (Morty et al., 1999a) and *T. evansi* (Morty et al., 2005a). Both *T. congolense* OPB (TcgOPB) and *T. evansi* OPB (TeOPB) are cytosolic, as with TbOPB, and both were similar in their activity to TbOPB, preferring substrates which filled both the P_1 and P_2 binding sites, particularly Gly, Lys, Arg, Phe or Leu as the P_2 amino acid. Thiol reducing agents also enhanced the activity of TcgOPB, thiol blocking agents inhibited it, and the same inhibitors were active as with TbOPB: DCI, AEBSF, DFP, PMSF, E64, antipain and leupeptin. DCI, AEBSF, PMSF, antipain and leupeptin also inhibit TeOPB.

TeOPB has been found in the bloodstream of infected rats, released from lysed parasites. As was found with TbOPB (Troeberg et al., 1996), TeOPB can cleave atrial natriuretic factor (ANF) after four arginine residues (Morty et al., 2005a). A correlation was found between reduced levels of ANF in infected rats over the course of infection and increased TeOPB activity. TeOPB led to a 300-fold reduction in the half life of ANF in plasma from infected rats. ANF controls blood volume and reduced levels in infected animals may contribute to the pathogenesis of trypanosomiasis (Morty et al., 2005a).

4.1.6 *Leishmania* Oligopeptidase B

A serine oligopeptidase, likely to be OPB, was described in *L. amazonensis* in 1998, though the peptidase had a molecular weight of 101 kDa, higher than OPBs so far described. It was inhibited by similar serine peptidase inhibitors (DIC, TLCK, AEBSF and to a lesser extent PMSF), and was specific for cleaving peptide

substrates, post-basic amino acids, but not cleaving proteins (de Andrade et al., 1998).

OPB was identified in *L. major* in 1999 (Morty et al., 1999a) and was subsequently annotated in the genomes of *L. major*, *L. infantum* and *L. braziliensis* (Ivens et al., 2005; Peacock et al., 2007).

OPB from *L. amazonensis* (LaOPB) was later cloned based on the sequence of *L. major* OPB (de Matos Guedes et al., 2007). It was sequenced and found to be 90% identical to *L. major* and *L. infantum* OPB and 84% identical to *L. braziliensis*, 67% identical to trypanosome OPBs and 37% identical to bacterial OPBs. As detailed above, C^{256} in *T. brucei* OPB is responsible for inhibition by thiol activating agents. This cysteine is a glycine residue in *Leishmania* OPBs as in TcOPB, suggesting that *Leishmania* OPBs will be resistant to iodoacetic acid and *N*-ethylmaleimide, as with TcOPB (de Matos Guedes et al., 2007).

4.1.6.1 Expression of OPB in *Leishmania*

OPB is a putative marker for the amastigote stage of the life cycle in *L. braziliensis*, having been found to be upregulated in amastigotes by a differential display screening and by quantitative PCR analysis of cDNA from the three life cycle stages of *L. braziliensis* (Gamboa et al., 2007). OPB was also found to have increased expression in amastigotes in a proteomic screen of *L. donovani* differentiating from metacyclic promastigotes to axenic amastigotes, reaching a peak of expression 24 hours after differentiation was initiated; physical transformation to the amastigote form occurs from around 15 hours after initiation (Rosenzweig et al., 2008).

4.1.7 Overview

The substrate preferences and inhibitory profile of OPBs from several species have been described above. It has been shown in these studies that cleavage of peptides post-arginine residues is a distinguishing feature of OPBs and they are inhibited by a number of serine peptidases, although often not by PMSF. This activity is exactly as was demonstrated for the cleavage of Bz-R-AMC by live *Leishmania* as described in Chapter 3, which suggests that the identification of OPB as being most likely to be responsible for the peptidase activity was probably

correct. However, as there are two other OPB-like peptidases in *L. major*, detailed characterisation of OPB is necessary to validate this suggestion.

4.2 Results

4.2.1 Expression of OPB in *Leishmania* species

Recombinant OPB was produced in *E. coli* and used to produce polyclonal antibodies in a sheep, which were then affinity purified (as described in Section 2.6.2). These antibodies were used for a western immunoblot using cell lysate of all three of the life cycle stages of *L. major* i.e., replicative promastigotes, infective metacyclic promastigotes and amastigotes purified from a lesion on an infected BALB/c mouse. OPB protein was found in all three stages (Figure 4.2).

OPB was also investigated in other *Leishmania* species. A western immunoblot was completed with cell lysate of both log and stationary phase promastigotes of *L. mexicana*, *L. infantum* and *L. donovani*. The anti-*L. major* OPB antibody successfully recognised the expression of OPB in the other species (Figure 4.3).

4.2.2 Localisation of OPB in fractionated cells

Purified anti-OPB antibody was used for western immunoblotting to identify the fraction containing OPB. *L. major* cells were sonicated with inhibitors that did not affect the cleavage of Bz-R-AMC by live cells, to prevent excessive degradation in the sonicate from peptidases released from organelles (E64-d, EDTA, 1,10 Phenanthroline and Pepstatin A). The supernatant, containing the soluble fraction, and the pellet, containing the membrane fraction, were separated as described in Section 2.1.6 and immunoblotted (Figure 4.4). The antibodies used were affinity purified anti-OPB, anti-EF-1α (a soluble protein) and anti-GP63 (a membrane protein). OPB was found to be in the soluble fraction of *L. major* (Figure 4.4).

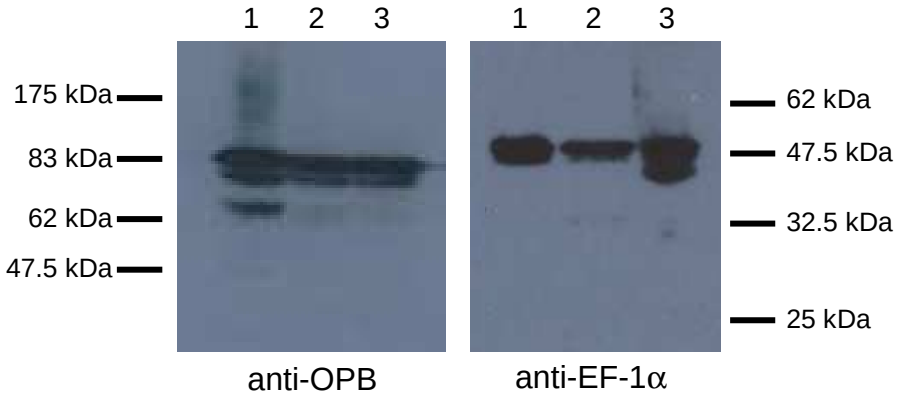


Figure 4.2 Western immunoblot showing the expression of OPB in the three life cycle stages of *L. major*. 1 = procyclic promastigotes, 2 = metacyclic promastigotes, 3 = amastigotes. A lysate of 5 x 10^6 cells was run in each lane. Purified anti-OPB antibody was applied at a 1 in 20,000 dilution and anti-*T. brucei* elongation factor 1α (EF-1α) antibody was applied at a 1 in 10,000 dilution. HRP-conjugated anti-sheep and anti-mouse secondary antibodies were applied at a 1 in 5,000 concentration. The chemiluminescence was detected in an Xomat processor.

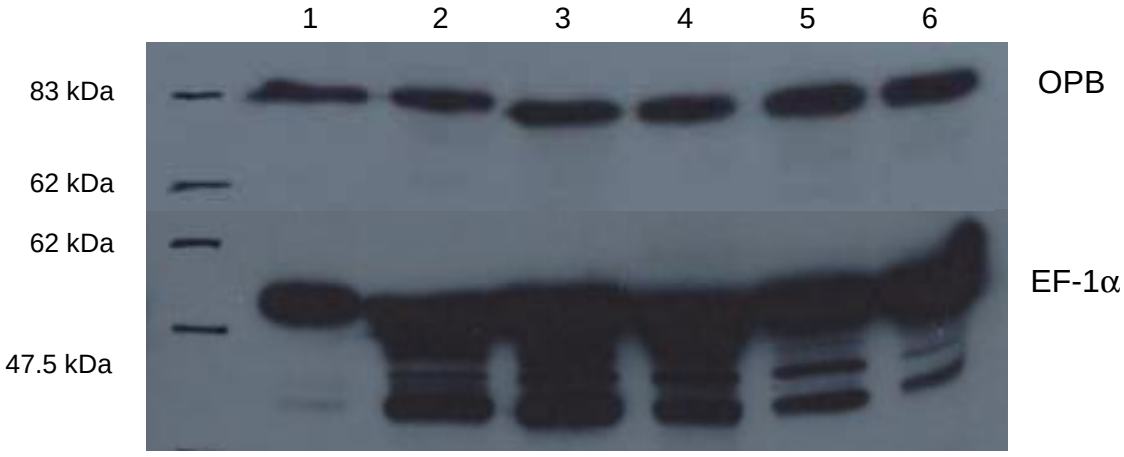


Figure 4.3 Western immunoblot showing expression of OPB in *L. mexicana, L. infantum* and *L. donovani* promastigotes. 1 = *L. mexicana* log phase, 2 = *L. mexicana* stationary phase, 3 = *L. infantum* log phase, 4 = *L. infantum* stationary phase, 5 = *L. donovani* log phase, 6 = *L. donovani* stationary phase. A lysate of 5 x 10^6 cells were run in each lane. Purified anti-OPB antibody was applied at a 1 in 20,000 dilution and anti-EF-1α antibody was applied at a 1 in 10,000 dilution. HRP-conjugated anti-sheep and anti-mouse secondary antibodies were applied at a 1 in 5,000 concentration. The chemiluminescence was detected in an Xomat processor.

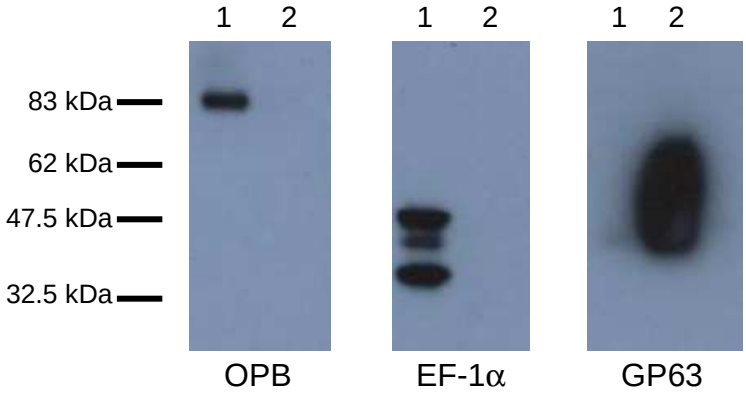


Figure 4.4 Western immunoblot to determine the location of OPB. OPB was localised to either soluble or membrane fraction of *L. major*. 1 = soluble fraction, 2 = pelleted fraction. Purified anti-OPB antibody was applied at a concentration of 1 in 20,000, with anti-EF-1α used at a concentration of 1 in 10,000 (as a control for the soluble fraction) and anti-GP63 used at a concentration of 1 in 100 (as a control for the membrane fraction). HRP-conjugated anti-sheep and anti-mouse secondary antibodies were applied at a 1 in 5,000 concentration. The chemiluminescence was detected in an Xomat processor.

4.2.3 Localisation of OPB by immunofluorescence analysis

For localisation, immunofluorescence analysis was undertaken on *L. major* promastigotes and *L. major* infected mouse macrophages and infected THP-1 cells.

4.2.3.1 Localisation in promastigotes

Purified anti-OPB antibody was used at two concentrations and co-localisation was carried out with concanavalin A as a marker for the flagellar pocket.

A 1 in 200 dilution of purified anti-OPB antibody was used initially, detecting the OPB with a rhodamine-conjugated secondary antibody, with DAPI staining to determine the location of DNA. OPB was apparently cytosolic, with a potential focus at the base of the flagellum, perhaps indicating the flagellar pocket. OPB was found in the flagellum, but appears to be excluded from the nucleus (Figure 4.5). A lower concentration of anti-OPB antibody was then used, a 1 in 500 dilution of primary antibody, which also appeared to have a slight focus in the region of the flagellar pocket (Figure 4.5C and D).

Only diffuse staining was found in secondary antibody only controls (Figure 4.5E) and the addition of exogenous recombinant OPB effectively blocked immuno-fluorescence using anti-OPB antibodies (Figure 4.5F).

Promastigotes were incubated with concanavalin A for 1 hour, the concanavalin A was washed off and the cells fixed and prepared for immunofluorescence as detailed in Sections 2.6.5.4. There was no co-localisation of the green concanavalin A with any focus of the rhodamine-detected OPB (Figure 4.6).

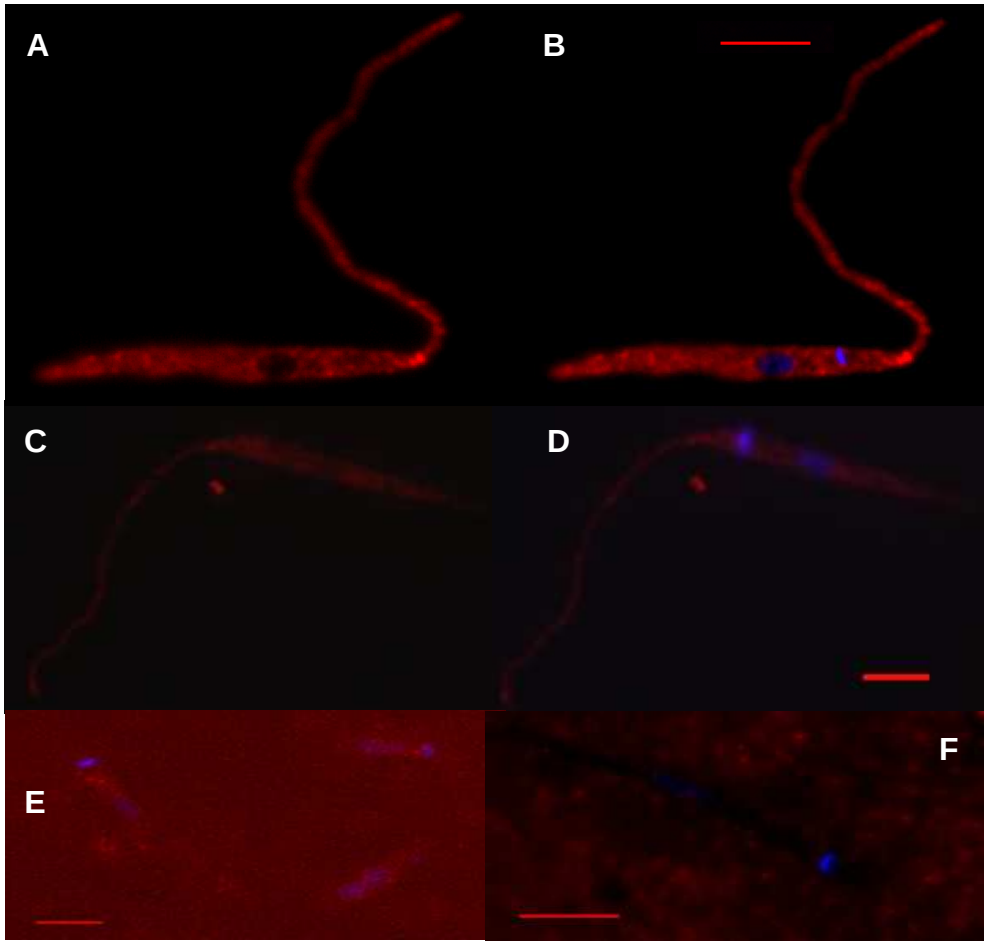


Figure 4.5 Localisation of OPB in *L. major*. 1 in 200 dilution of primary antibody was used for A, B and F, 1 in 500 dilution for C and D, rhodamine secondary antibody alone for E and recombinant OPB was added to promastigotes prior to immunofluorescence analysis for F. A and C = anti-OPB antibody stained with rhodamine secondary antibody at a 1 in 1000 dilution, B, D, E and F = anti-OPB antibody staining merged with DAPI staining of nucleus and kinetoplast. 100 ms exposure was used. Scale = 5 µm.

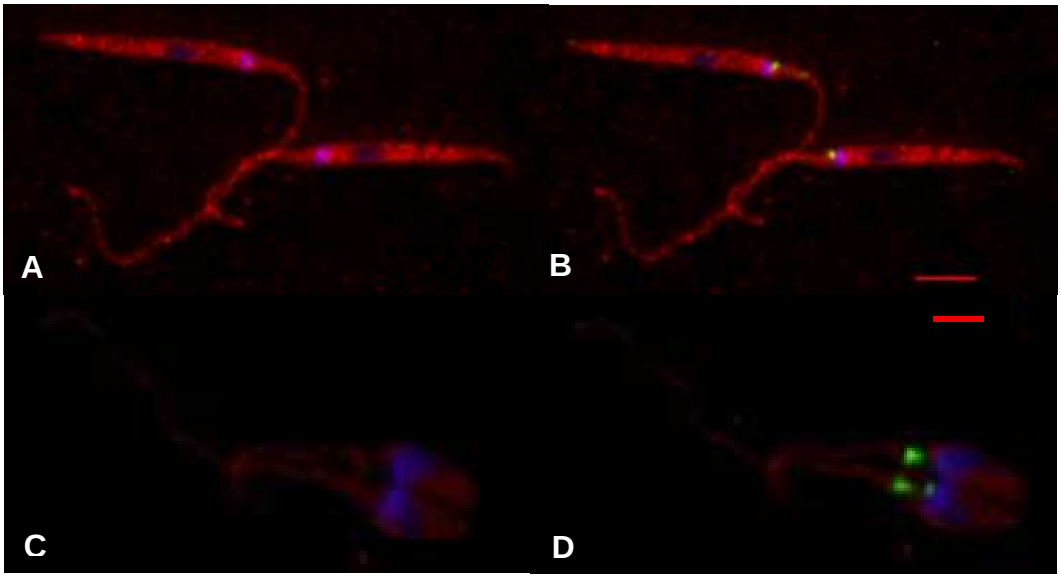


Figure 4.6 Comparison of OPB localisation with concanavalin A staining of flagellar pocket. A and B = 1 in 200 dilution of primary antibody, C and D = 1 in 500 dilution of primary antibody. A and C = anti-OPB antibody stained with rhodamine secondary antibody merged with DAPI staining of nucleus and kinetoplast, B and D = anti-OPB antibody merged with DAPI and co-localised with concanavalin A. 100 ms exposure used for rhodamine and DAPI and 45 ms for concanavalin A. Scale = 5 µm.

4.2.3.2 Localisation in intracellular amastigotes

Release of OPB from the cell was investigated using immunofluorescence of PEM infected with *L. major*. The amastigotes could be detected through the use of DAPI staining of the nucleus and kinetoplast, but the localisation of *Leishmania* OPB could not be determined as there was cross-reaction of the OPB antibody with proteins in the macrophage, with fluorescent signal throughout the cell. This was confirmed by controls using uninfected macrophages, where the same distribution of fluorescence was found (Figure 4.7A and B).

The monocyte line THP-1 was differentiated to attached cells (as detailed in Section 2.1.7) and cells were also used for immunofluorescence, either infected or uninfected with *L. major*. As with the infected PEM the amastigotes could be detected through the DAPI staining of DNA. However, proteins within the THP-1 cell were also detected by the anti-OPB antibody, with fluorescence throughout the cell. Again, this was confirmed as the uninfected cells had the same pattern of fluorescence (Figure 4.7C and D).

The cross-reactive protein detected by immunofluorescence in PEM was also detected by western immunoblotting at over 175 kDa, though no extra protein was detected in the THP-1 cells (Figure 4.8). *L. major* OPB was just detected in the infected PEM (lane 1). A protein of the correct size for *L. major* OPB was detected in the infected THP-1 cells (lane 3), with the cells presumably more heavily infected than the PEM given the relative strengths of the signal (lane 3 versus lane 1).

4.2.4 Localisation of the site of cleavage of Bz-R-AMC

To investigate further whether OPB could be responsible for the cleavage of Bz-R-AMC in live promastigotes, the hydrolysis was localised by visualisation on a fluorescence microscope. Bz-R-AMC was added to promastigotes for a period of 10 minutes, then the cells were washed with PBS and the AMC was visualised using an excitation wavelength of 365 nm and an emission wavelength of 450 nm. The AMC was shown to appear in the cytosol following cleavage (Figure 4.9).

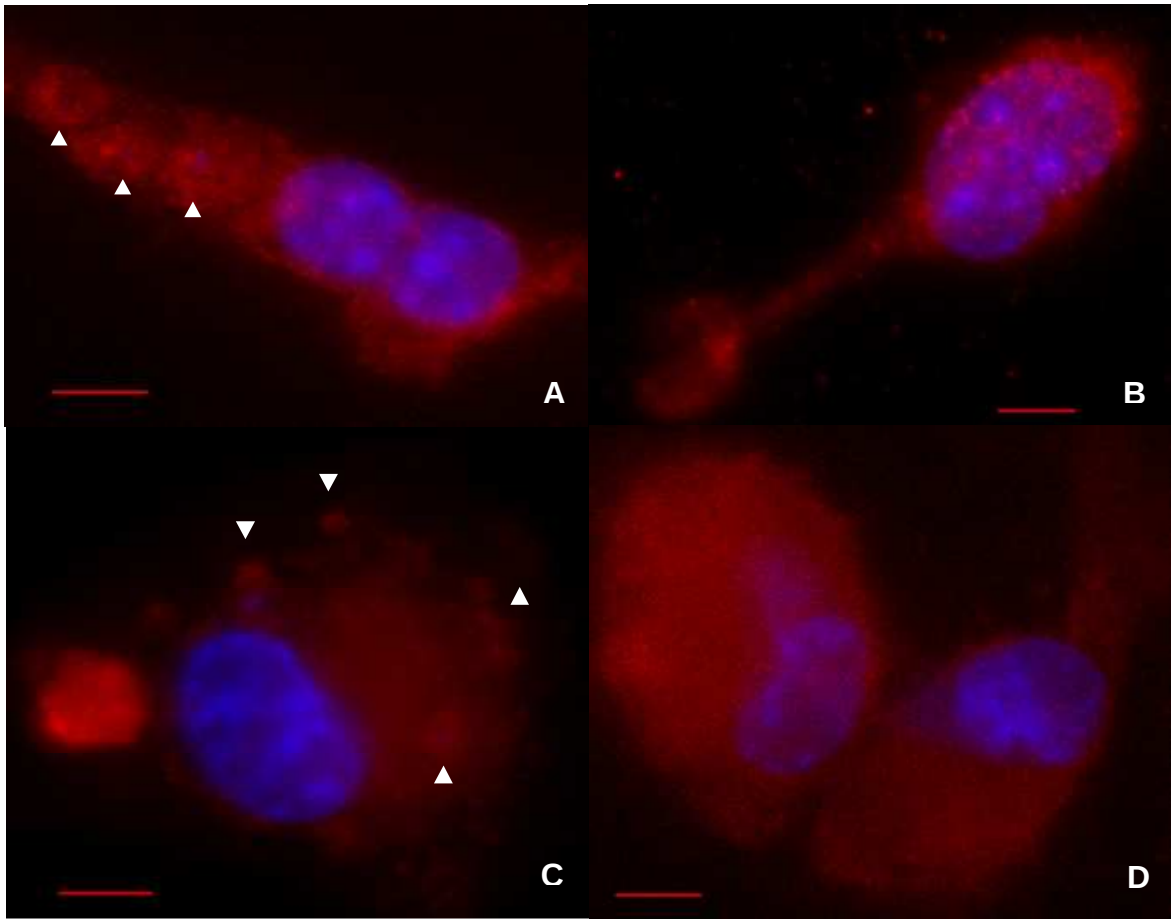


Figure 4.7 Immunofluorescence of PEM and THP-1 cells, using anti-OPB antibody. A = PEM infected with *L. major* for 4 days, B = uninfected PEM. For A and B a 1 in 200 dilution of primary antibody was used, with DAPI staining of DNA and an exposure time of 100 ms for both. C = THP-1 cells infected with *L. major* for 4 days, D = uninfected THP-1 cells. For C and D a 1 in 1000 dilution of primary antibody was used, with DAPI staining of DNA and an exposure time of 50 ms for both. White arrowheads indicate position of amastigotes. Scale = 5 µm.

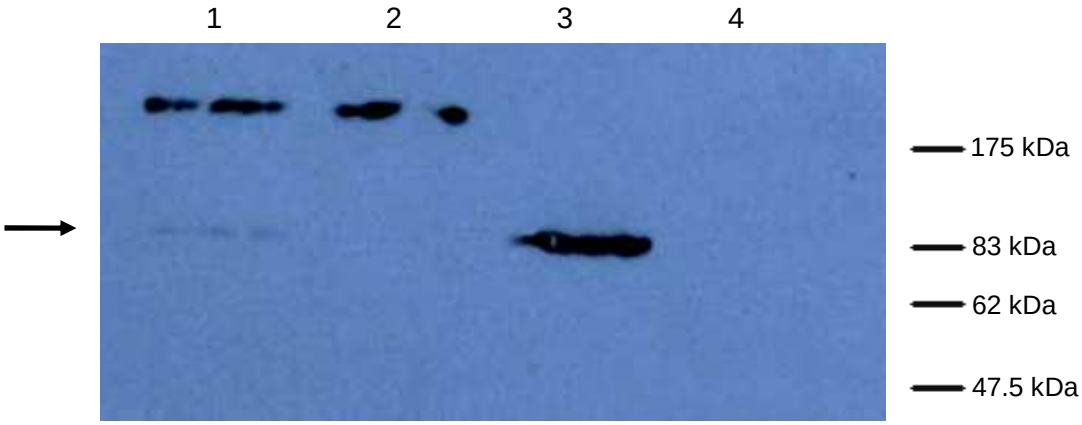


Figure 4.8 Western immunoblot of PEM and THP-1 cells infected and uninfected with *L. major* stationary phase promastigotes. 1 = PEM infected with *L. major* for 5 days, 2 = uninfected PEM, 3 = THP-1 cells infected with *L. major* for 6 days, 4 = uninfected THP-1 cells. Purified anti-OPB antibody was used. 8 x 10^4 macrophage cells were run per lane. Anti-OPB antibody was applied at a concentration of 1 in 20,000. HRP-conjugated anti-sheep secondary antibody was applied at a 1 in 5,000 concentration. The chemiluminescence was detected in an Xomat processor. Arrow marks position of *L. major* OPB on blot.

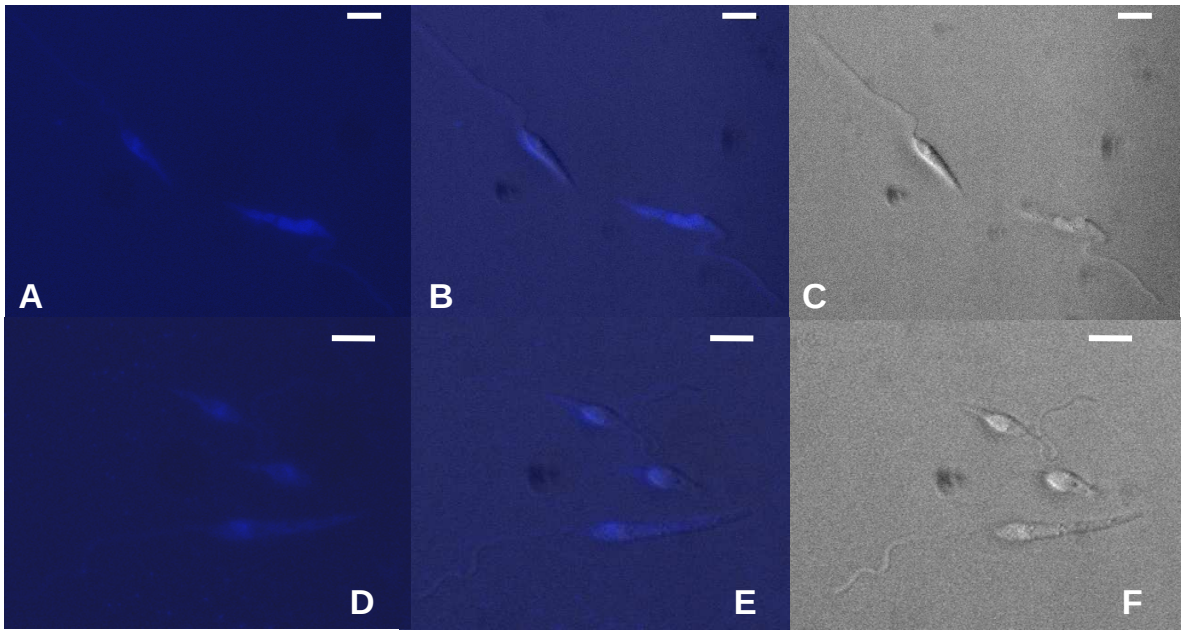


Figure 4.9 Cleavage of 50 µM Bz-R-AMC localised by fluorescent microscopy. Two examples of representative promastigotes. A and D = AMC fluorescence alone, B and E = AMC fluorescence merged with phase, C and F = phase alone. 50 ms exposure was used for the AMC. Scale = 5 µm.

4.2.5 Immunoprecipitation of OPB from sonicated *L. major*

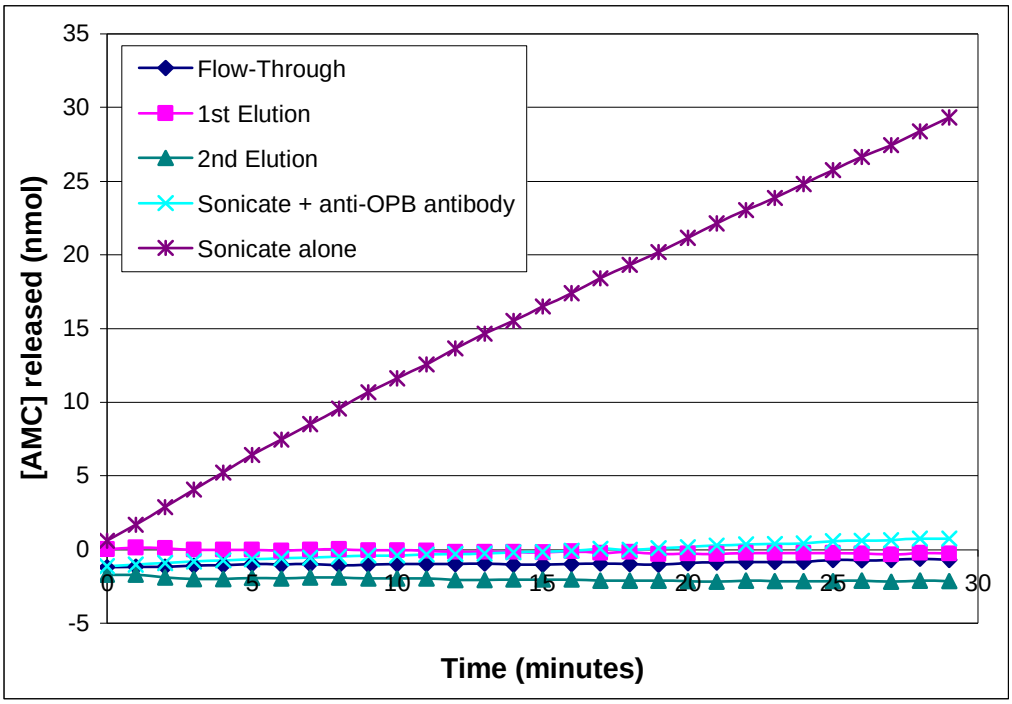
L. major cells were sonicated with the inhibitors that did not affect the cleavage of Bz-R-AMC by live cells (E64-d, EDTA, 1,10 Phenanthroline and Pepstatin A), to prevent excessive cleavage in the sonicate from peptidases released from organelles. The *L. major* sonicate was incubated overnight with either anti-OPB antibody or pre-immune serum (P.I.S.), as a control. The mixtures were run through protein G columns and three fractions collected: the flow-through from the column and two elutions. The flow-through should contain all proteins not bound by the antibody and the elutions from the columns contain the antibody and any protein they have bound. These fractions, the sonicate bound to either anti-OPB antibody or P.I.S. and the sonicate alone were used to investigate the cleavage of Bz-R-AMC and for western immunoblotting, using anti-OPB antibody, to identify the fraction containing OPB.

The sonicate alone, prior to binding of either antibody or serum, was able to cleave Bz-R-AMC effectively. Using the fractions from the column with the anti-OPB antibody-bound sonicate run through it, there was no cleavage of Bz-R-AMC by the flow-through from the column. The peptidase which cleaves Bz-R-AMC was thus retained on the column. There was also no cleavage activity in either of the two elutions or the sonicate that had been bound to the anti-OPB antibody, but not run through the column (Figure 4.10A), given this, the anti-OPB antibody must bind OPB in such a way as to inhibit its activity.

With the column with the P.I.S.-bound sonicate run through it, both the flow-through from the column and the sonicate bound to serum but not put through the column were still able to cleave Bz-R-AMC. Neither elution from the column cleaved Bz-R-AMC (Figure 4.10B). This shows the P.I.S. was not able to bind to the peptidase which cleaves Bz-R-AMC.

A western immunoblot to show presence of OPB was completed by Dr Nicholas D. Bland (University of Glasgow), using the fractions from the columns and the sonicate bound overnight to antibody or pre-immune serum (Figure 4.11). With the fractions from the column which had anti-OPB antibody-bound sonicate run through it, OPB was found in the sonicate bound to antibody fraction and in the first elution.

A



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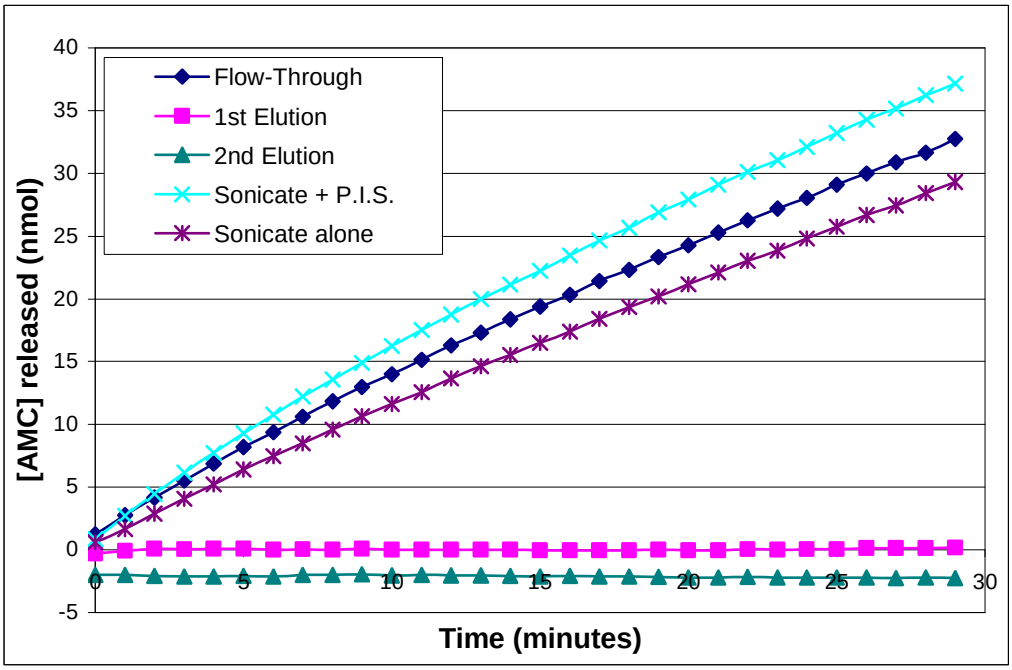


Figure 4.10 OPB Activity after immuno-precipitation. A = activity after immuno-precipitation of sonicate bound to anti-OPB antibody, B = activity after immuno-precipitation of sonicate bound to pre-immune serum (P.I.S.). Flow-through = run through of sonicate from column, 1st and 2nd elutions = elutions from column, sonicate + anti-OPB antibody = *L. major* sonicate bound overnight to anti-OPB antibody, sonicate + P.I.S. = *L. major* sonicate bound overnight to pre-immune serum, and sonicate alone = sonicate not bound to anything. A volume of liquid equivalent to the sonicate of 3 x 10^5 stationary phase cells were assayed for each fraction. Representative graphs of two experiments are shown.

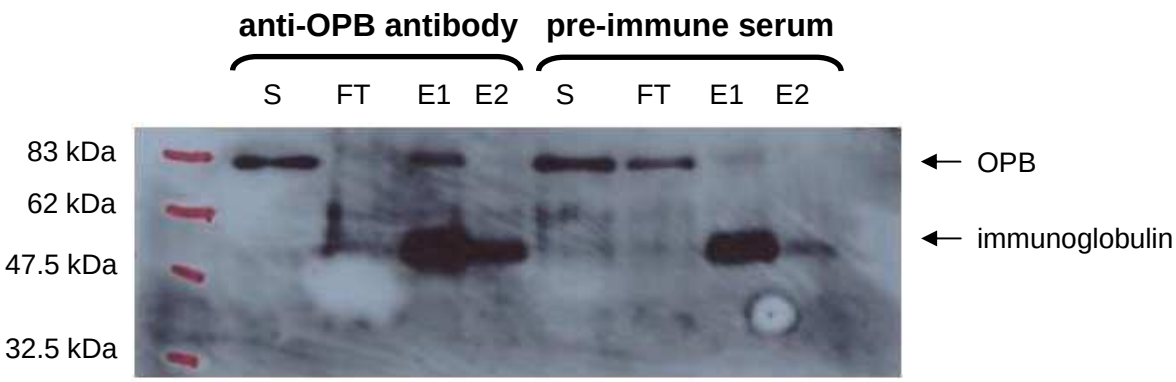


Figure 4.11 Western blot of fractions from immuno-precipitation with anti-OPB antibody. Fractions are: sonicate bound to serum (S), flow-through from column (FT), elution 1 from column (E1) and elution 2 (E2). Anti-OPB antibody was applied at a concentration of 1 in 20,000. HRP-conjugated anti-sheep secondary antibody was applied at a 1 in 5,000 concentration. The chemiluminescence was detected in an Xomat processor.

This confirms that the anti-OPB antibody had bound the OPB in the *L. major* sonicate and that the antibodies were retained by the protein G column, as there was no OPB present in the flow-through fraction. In contrast, the fractions from the column which had pre-immune serum-bound sonicate run through it only contained OPB in the sonicate bound to serum fraction and in the flow-through. The antibodies in the pre-immune serum were not specific to OPB and so the OPB was not retained on the column.

4.2.6 Inhibitors of recombinant OPB in live cell tests

Polyamine compounds (CMR compounds) were designed within the Chemistry Department at the University of Glasgow as potential anti-cancer agents. The members of this series of compounds with side chains with similarity to arginine, the preferred P_1 amino acid for OPB, were identified as potential OPB inhibitors. In addition, other compound in this series with the same polyamine ring structure but with different side chains, were identified as useful controls. Their structures and their K_is against recombinant OPB are shown in Figure 4.12. Their K_is were determined by Dr. Nicholas D. Bland of the University of Glasgow.

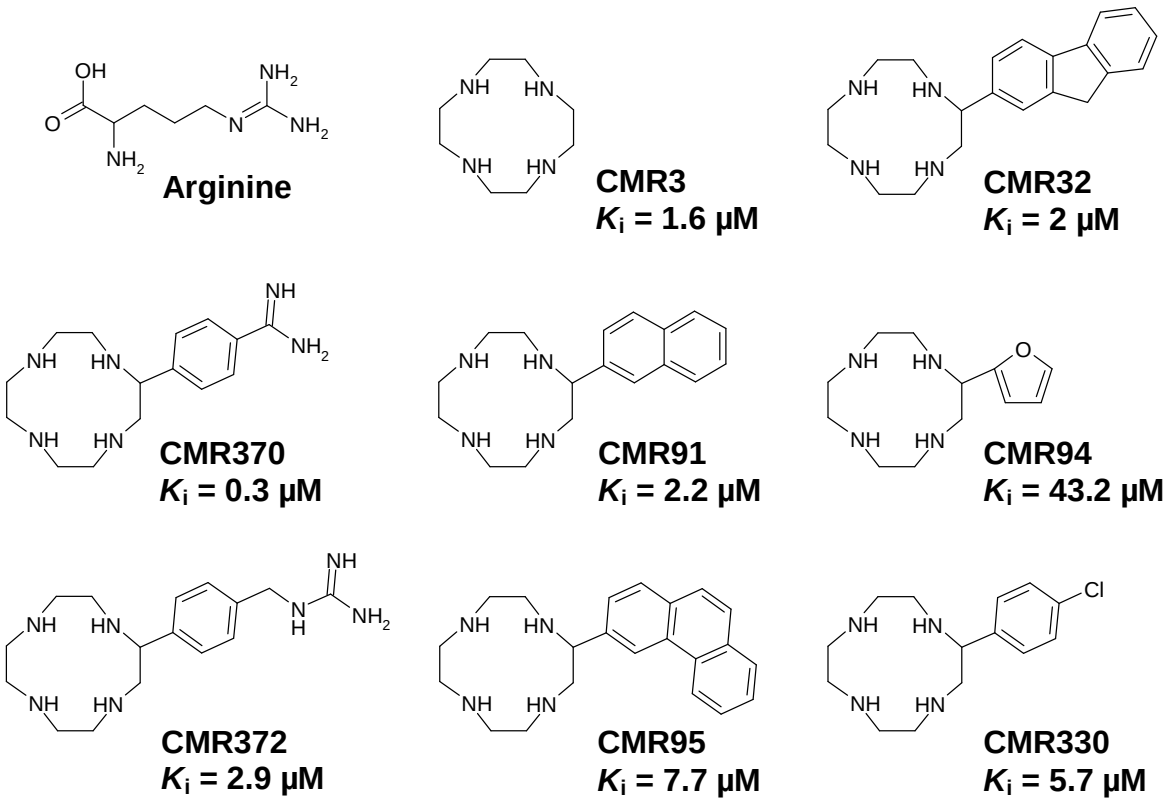


Figure 4.12 Structures of polyamine compounds (CMR compounds), compared to the amino acid arginine. These were tested for inhibition of recombinant OPB and the resultant K_is are shown.

The best inhibitor of recombinant OPB was CMR370, one of the compounds with the arginine-like side chain. However, other compounds without this side chain were as good or better inhibitors of recombinant OPB than compound CMR372, which also has an arginine-like side chain. This suggests that the compounds may not be inhibiting simply via binding the active site that accepts arginine.

The effect of the CMR compounds on the growth of *L. major* promastigotes was determined by alamar blue assay (Table 4.1). CMR inhibitors affected the growth of parasites. Although CMR370 was one of the best inhibitors, other compounds without arginine-like side chains were also as effective anti-leishmanial compounds.

The LD_{50} values were also determined for mouse PEM and the human cell line HEK, by alamar blue assays, prior to being used for infected macrophage assays (Table 4.1). The HEK cell LD_{50} values were generated by Dr. Charles Ebikeme (University of Glasgow).

Table 4.1 Mean LD_{50} found for CMR compounds against *L. major* promastigotes and mammalian cells by alamar blue assay.

Inhibitor	LD_{50} for *L. major* promastigotes	LD_{50} for mouse PEM	LD_{50} for HEK cells
CMR32	5.4 µM ± 1.3	21.9 µM	26.0 µM
CMR330	8.4 µM ± 0.3	104 µM	181.2 µM
CMR95	10.1 µM ± 1.0	33.2 µM	24.9 µM
CMR370	11.0 µM ± 1.1	133 µM	>200 µM
CMR91	27.8 µM ± 4.5	56.6 µM	>200 µM
CMR3	33.9 µM ± 4.5	104 µM	5.0 µM
CMR372	115.3 µM ± 29.6	>1000 µM	>200 µM
CMR94	>1 mM	nd	>200 µM

Several of the CMR compounds had very similar LD_{50} values for both *L. major* and mammalian cells, the difference being only between 2- and 3-fold. However, the best anti-recombinant OPB compound, CMR370, had a 10-fold higher LD_{50} value against PEM compared to *L. major* and so this compound was chosen for infected PEM assays.

Since CMR 370 also affected the growth of macrophages, concentrations from 100 µM down to 2.5 µM were used for infected PEM assays. However, at this concentration the amastigotes appeared to disintegrate, but not to be removed (Figure 4.13 A). The disintegration also occurred at lower concentrations (Figure 4.13 B), though not with untreated cells (Figure 4.13 C). The macrophage cytoplasm was obscured to such an extent that it was impossible to determine if whole amastigotes were also present.

CMR3 and CMR370 were used to inhibit the cleavage of Bz-R-AMC by *L. mexicana* and *L. major* WT promastigotes. CMR3 had no effect on activity of the enzyme (Figure 4.14), whilst CMR370 did inhibit (Figure 4.15 and Table 4.2). Due to the inherent fluorescence of CMR370 the AMC values released are artificially high for the three highest concentrations used.

4.3 Discussion

Western blot analysis with specific anti-OPB antibody has shown that OPB is equally expressed in all life cycle stages in *L. major* (Figure 4.2). The amastigote expression was corroborated as OPB has previously been shown to be present in *L. braziliensis* and *L. donovani* amastigotes (Gamboa et al., 2007; Rosenzweig et al., 2008). OPB has also been found to be expressed in both promastigotes and axenic amastigotes of *L. amazonensis* (de Matos Guedes et al., 2007).

Anti-*L. major* OPB antibodies can also successfully recognise OPB from other species (Figure 4.3), which is unsurprising given that the *Leishmania* OPB protein sequences currently known are at least 94% identical to one another.

OPB was localised to the soluble fraction (Figure 4.4) and subsequently to the cytosol by immunofluorescence (Figure 4.5). This apparent cytosolic location is consistent with that found for other kinetoplastid OPBs (Burleigh et al., 1997; Morty et al., 2005a; Rea and Fulop, 2006). A focus at the base of the flagellum suggested that OPB might be located in the flagellar pocket and so secreted, but failure to co-localise with the flagellar pocket marker, concanavalin A, suggests this may be an artefact of the immunofluorescence staining (Figure 4.6). However, even though OPB does not contain a signal sequence for secretion it has been found in the secretome of *L. donovani* (Silverman et al., 2008). One

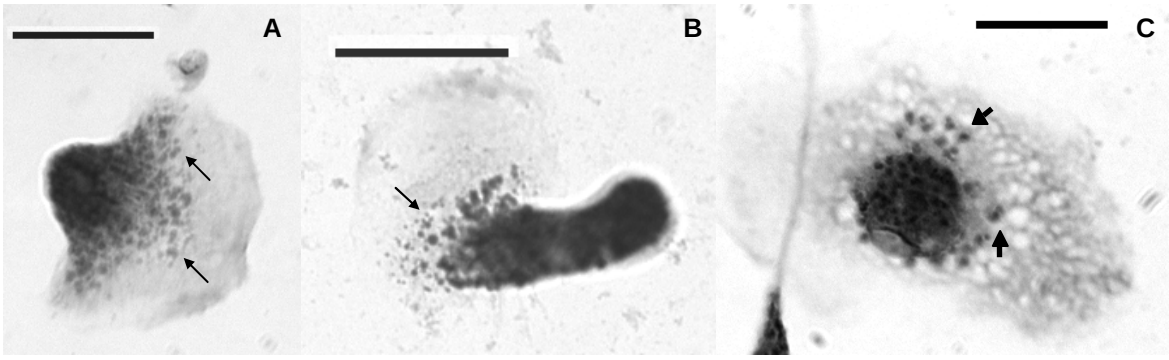
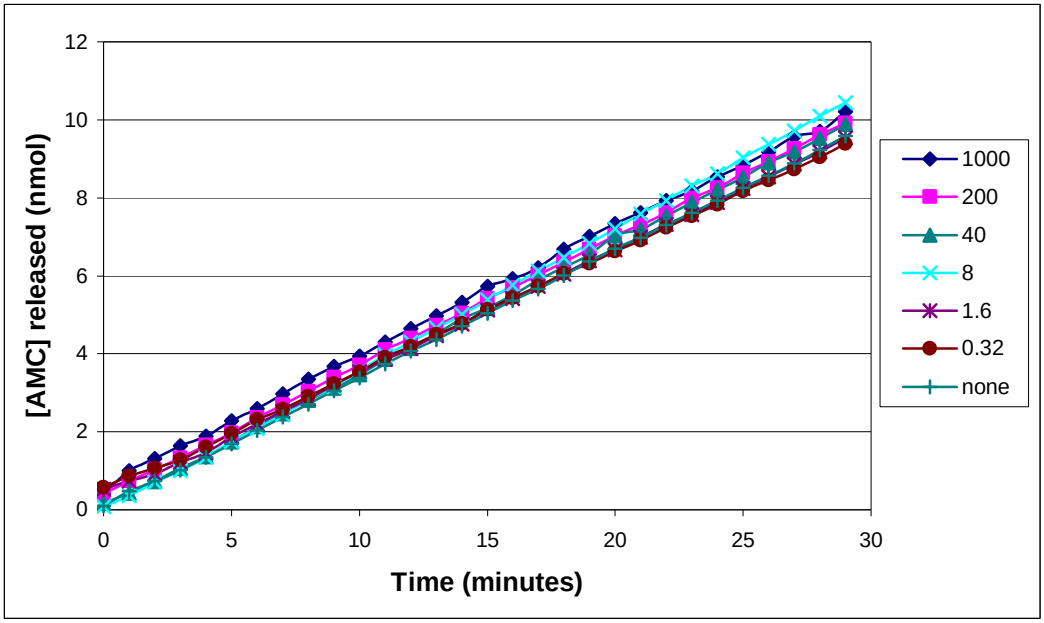


Figure 4.13 Effect of CMR 370 on PEM infected with *L. major*. A = cells treated with 100 µM CMR 370; B = cells treated with 22 µM CMR 370; C = untreated control cells. Cells were infected at a ratio of 8 promastigotes to one macrophage overnight, treated the following day and Giemsa's stained 5 days later. Example disintegrated amastigotes are indicated by thin arrows, whole amastigotes by thick arrows. Scale = 10 µm.

A



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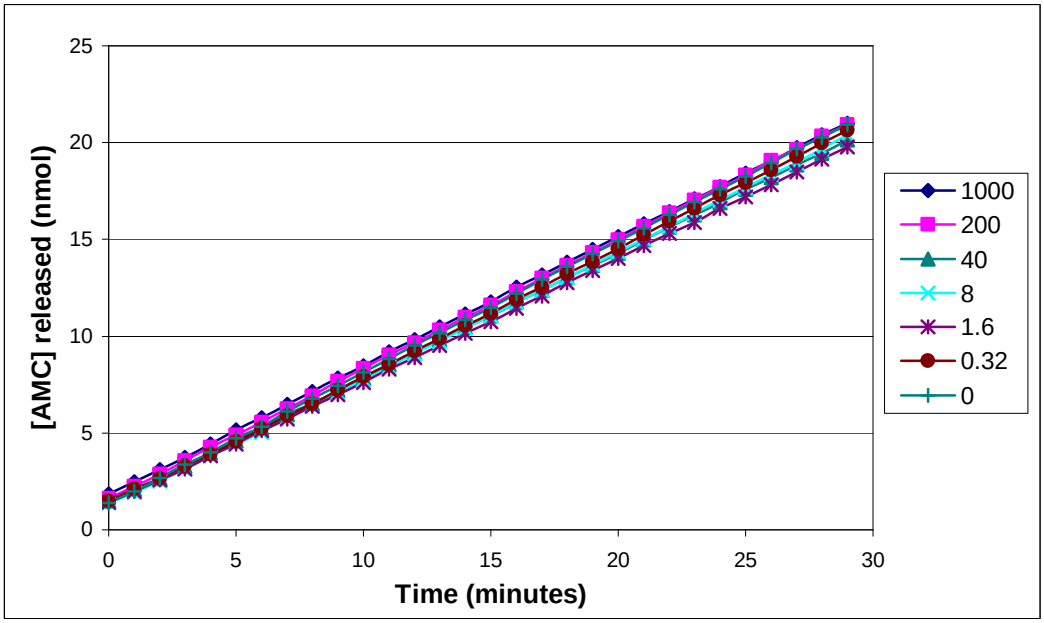
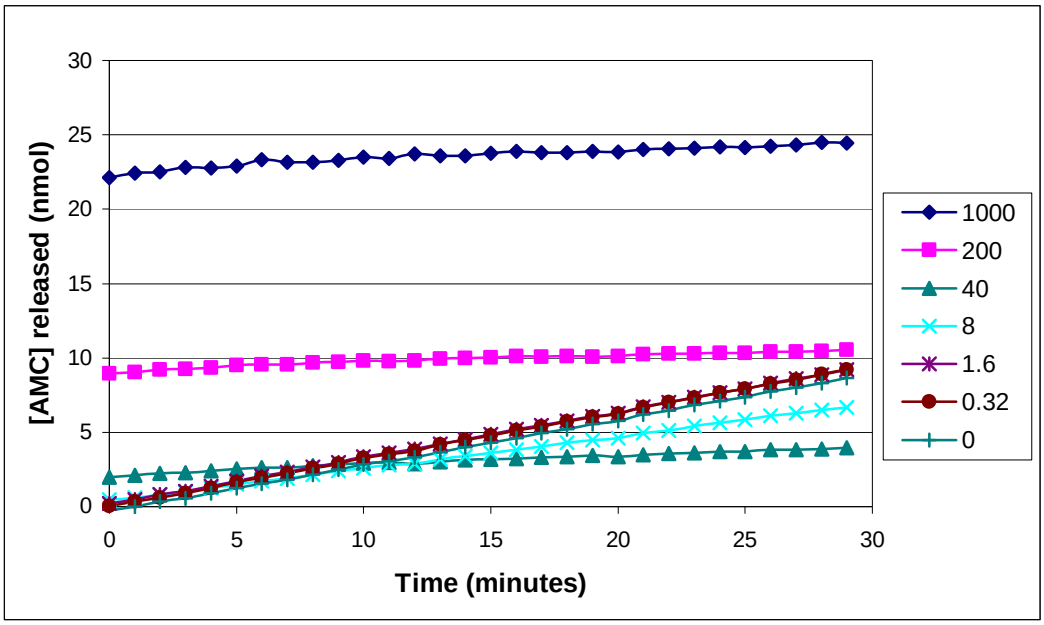


Figure 4.14 Effect of CMR 3 on the cleavage of 5 µM Bz-R-AMC. A = *L. major* WT stationary phase promastigotes, B = *L. mexicana* WT stationary phase promastigotes. Legend indicates concentration of CMR3 used (µM). 6 x 10^5 cells were used per experiment. Representative graphs of 3 experiments are shown.

A



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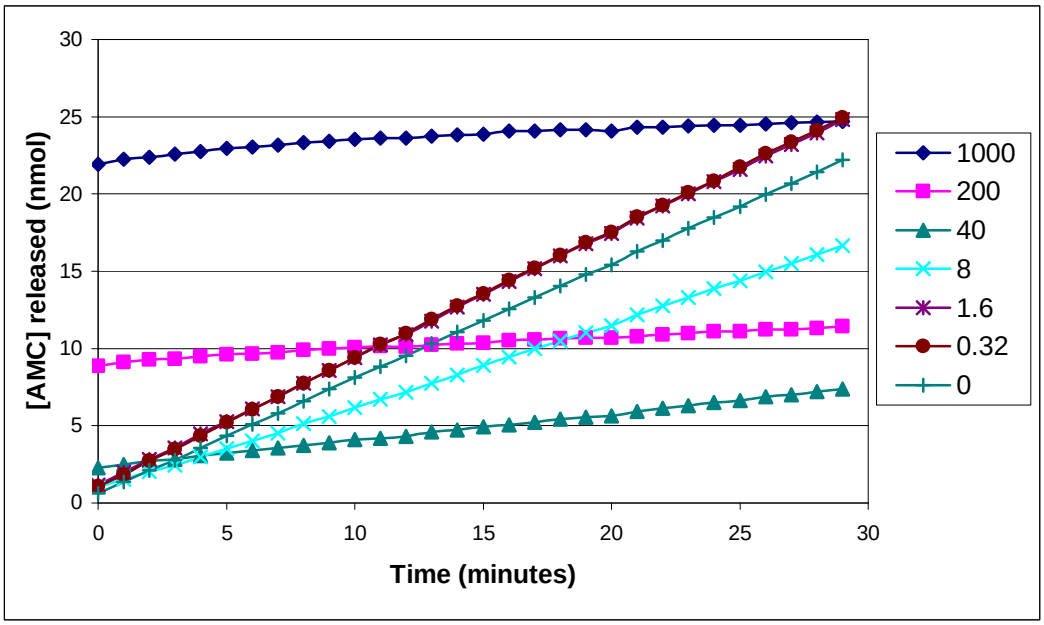


Figure 4.15 Effect of CMR 370 on the cleavage of 5 µM Bz-R-AMC. A = *L. major* WT stationary phase promastigotes, B = *L. mexicana* WT stationary phase promastigotes. At high concentrations CMR370 is fluorescent, affecting the values for AMC release. Legend indicates concentration of CMR370 used (µM). 6 x 10^5 cells were used per experiment. Representative graphs of 3 experiments are shown.

Table 4.2 IC_{50} values for inhibition of Bz-R-AMC cleavage by CMR 370.

	Mean IC_{50} ± SEM
***L. major* stationary phase promastigotes**	7.41 µM ± 1.62
***L. mexicana* stationary phase promastigotes**	11.19 µM ± 2.75

other report of an OPB being released has been made, for *T. cruzi* OPB (Fernandes et al., 2005), though there is no evidence for secretion of other OPBs, including African trypanosome OPBs (Morty et al., 2001; Morty et al., 2005a).

Localisation of OPB in amastigotes within macrophages, or of potential secretion of OPB into the macrophage, was not possible due to cross reaction of the anti-OPB antibody with a protein within the macrophages (Figure 4.7), but it is not unreasonable to predict that it is cytosolic as well. The cross reactive protein could be another member of the POP family, which is expressed in mammalian cells, as the family appear to show structural similarity (Gerczei et al., 2000) and the POP family member prolyl endopeptidase has been found to be present cytosolically, in the perinuclear region and in association with the microtubule cytoskeleton in cultured glioma cell lines (Schulz et al., 2005).

The cytosolic location of OPB suggests that it could be responsible for the cleavage of Bz-R-AMC. This substrate probably enters *L. major* by diffusion through the membrane, which would lead to it being located in the cytosol and therefore available to the enzyme. The apparent co-localisation of OPB and Bz-R-AMC to the cytosol was confirmed by visualising the appearance of AMC fluorescence in the cell upon application of Bz-R-AMC to promastigotes (Figure 4.9).

Cleavage of Bz-R-AMC occurring in the same location as OPB was consistent with OPB being responsible for the cleavage. This was confirmed when immunoprecipitation of *L. major* sonicate with anti-OPB antibody removed peptidase activity from the sonicate (Figure 4.10). The anti-OPB antibody also prevented hydrolysis, as has previously been shown to occur with anti-*T. cruzi*, anti-*T. brucei* and anti-*T. evansi* OPB antibodies (Burleigh et al., 1997; Morty et al., 2001; Morty et al., 2005a). As antibodies are large multidomain proteins, they would be unable to access the active site of OPB due to the β-propeller and so are unlikely to block the active site directly, meaning it is more likely that one of the epitopes recognised by the polyclonal antibody is around the entrance of the β-propeller pore, thus preventing substrate access and catalysis.

Since OPB inhibitors have previously been shown to have trypanocidal activity (Morty et al., 2000), a number of potential OPB inhibitors were screened for OPB inhibitory activity and for anti-leishmanial activity. OPB is a promising drug target,

being absent from mammalian genomes and with antipain, an inhibitor of OPB, being recognised as an anti-leishmanial agent (Coombs et al., 1982; Coombs and Baxter, 1984).

The most active polyamine compound against recombinant OPB, CMR370 (Figure 4.12), had a low micromolar LD_{50} against *L. major* promastigotes (Table 4.1). However, several of the other polyamine compounds, of varying effectiveness at inhibiting recombinant OPB, were as effective or even more effective as anti-leishmanials. This suggests that there are other targets for these compounds within promastigotes, which, in hindsight, may not be unexpected as they were not designed specifically to inhibit OPB, but for testing as anti-cancer agents.

Many of the polyamine compounds were not specific in their anti-leishmanial properties. The compounds killed mouse PEM and HEK cells with LD_{50} values only 2 to 4-fold higher than those against *L. major* promastigotes (Table 4.1). Only CMR370 showed significant selectivity, with a 10-fold difference between its anti-leishmanial activity over its anti-mammalian cell activity. As such, amastigote killing assays were attempted in infected macrophages. CMR370 appears to affect the ability of macrophages to completely remove amastigotes, as drugged macrophages had what appeared to be large numbers of disintegrated amastigotes within their cytoplasm, even with levels of drug 6-fold lower than the PEM LD_{50} (Figure 4.13). This suggests that there is a target of CMR370 within the macrophage whose disruption leads to an inadequate clearing of amastigotes. The obscuring of the macrophage cytoplasm by disintegrated amastigotes meant it was impossible to be sure that whole amastigotes were not present, but assuming complete disintegration of amastigotes was occurring at high levels of CMR370 then this may be sufficient for anti-leishmanial action.

CMR370 was an inhibitor of the activity of OPB in live cell Bz-R-AMC cleavage assays (Figure 4.15), with a similar micromolar IC_{50} value as that of the anti-leishmanial LD_{50} (Table 4.2). Given this similarity, this compound may therefore be acting directly as an anti-OPB agent in promastigotes.

4.3.1 Conclusions

OPB has been demonstrated to be a cytosolic protein present throughout the life cycle in *Leishmania*. Localisation of OPB and the site of cleavage of Bz-R-AMC suggested OPB was the enzyme responsible for this activity. This was confirmed by determining that no cleavage occurred when OPB was immunoprecipitated from *L. major* sonicate.

OPB appears to be suitable as a drug target, being absent from mammalian species and with OPB inhibitors being able to kill promastigote *L. major*, and appearing to lead to disintegration of amastigotes within infected macrophages. Genetic manipulation of OPB in *L. major* to evaluate it as a drug target has been carried out and will be discussed in Chapter 5.

Chapter 5

GENETIC MANIPULATION OF *L. MAJOR* OPB

5.1 Introduction

5.1.1 Targeted gene replacement in *Leishmania*

Genetic manipulation involves the replacement of specific genes using constructs designed to exploit homologous recombination in *Leishmania*. These typically contain an antibiotic resistance gene (for examples, see Section 5.1.3), to allow selection, flanked by regions of DNA specific to the gene under study. The gene specific DNA either flanks or includes part of the open reading frame of the gene of interest in order to target the construct to the desired locus to replace or disrupt the gene (Clayton, 1999; Beverley, 2003). DNA is digested to leave just the flanks and the resistance gene, then introduced into *Leishmania* by electroporation. As *Leishmania* is a diploid organism two rounds of homologous recombination using different selectable markers are necessary to delete both alleles of a gene (Cruz et al., 1991).

Early studies indicated that a flank of around 200 bp was sufficient for gene replacement to occur successfully in *L. enriettii* (Tobin and Wirth, 1992). Subsequent work in *L. tarentolae* found that flanks of around 1 kb had the highest efficiency of replacement, with a minimum size of 150-200 bp being required for recombination. The successful integration of constructs designed for *L. tarentolae* into either *L. donovani* or *L. major* or from either of the latter two into *L. tarentolae* were highly dependent on the homology of the sequences in the three species; sequences with 86 or 87% homology could not recombine (Papadopoulou and Dumas, 1997).

For targeted gene replacement to work in *Leishmania*, homologous recombination is necessary. This was proven to occur in *Leishmania* in 1991 by the Wirth laboratory with *L. enriettii*. They transfected *L. enriettii* with two plasmids with independent mutations in different regions of the chloramphenicol acetyl-transferase (*CAT*) gene, which each caused a lack of activity. Recombination between the plasmids was shown, when CAT activity was found. The recombination was confirmed by Southern blotting and PCR. Recombination was also demonstrated between a different pair of plasmids as well, one carrying the *NEO* gene and the other the *CAT* gene (Tobin et al., 1991).

Targeted gene replacement in *Leishmania* was first illustrated in avirulent *L. major* in 1990, when a construct containing the neomycin phosphotransferase (*NEO)* gene was introduced into the dihydrofolate reductase-thymidine synthase (*DHFR-TS*) gene locus by homologous recombination using a construct containing the flanking sequences of *DHFR-TS* (Cruz and Beverley, 1990). Both alleles could be sequentially replaced using this construct and one containing hygromycin B phosphotransferase (*HYG*), if thymidine was added for growth (Cruz et al., 1991).

Targeted gene replacement was then demonstrated in *L. enriettii* by integration of a construct containing *NEO*, flanked by α-tubulin intergenic regions and coding sequences, into the α-tubulin locus (Tobin and Wirth, 1992). Subsequently, either of the two arrays of α-tubulin genes present in *L. enriettii* could be deleted either singly or together, provided an ectopic copy was added to maintain the viability of the parasites (Curotto de Lafaille and Wirth, 1992).

Targeted gene replacement has subsequently been used extensively by many laboratories to disrupt or remove both single genes and multi-copy gene arrays. Individual genes targeted within the Coombs and Mottram laboratories include the cysteine peptidases *CPA* and *CPC* (Souza et al., 1994; Bart et al., 1997) and *GPI8* (GPI:protein transamidase 8; Hilley et al., 2000). The gene array of the 19-copy cysteine peptidase *CPB* was also deleted by this method (Mottram et al., 1996b).

Essential genes can be identified by this technique as the gene will be retained by the parasite post-transfection. *Leishmania* have been found to use several mechanisms to survive selection with two antibiotics and maintain a copy of the original gene. Attempts to knock out *DHFR-TS* in virulent *L. major* had three results: i) the DNA content was doubled in some cells, to tetraploidy, maintaining two wild-type chromosomes and two chromosomes each with one resistance gene; ii) aneuploid lines, with only one extra chromosome (containing the wild-type gene), were generated; and iii) diploid cells, with insertion of the antibiotic resistance genes without replacement of the wild-type locus were found (Cruz et al., 1993). Cells with either triploid or tetraploid DNA content were also found when it was attempted to delete *CRK1* in *L. mexicana*. Both alleles could only be removed without altering ploidy if an ectopic gene copy was also expressed (Mottram et al., 1996a). Genomic rearrangements to maintain a wild-type gene have been found also in *L. donovani* when it was attempted to knock out the

trypanothione reductase gene, with an extra copy found on a different chromosome (Dumas et al., 1997).

5.1.2 Ectopic expression of genes in *Leishmania*

Extrachromosomal expression of genes in *Leishmania* has been possible since 1989, when it was demonstrated that *CAT* could be transiently expressed on an extrachromosomal element in *L. enriettii*, *L. major* and *L. braziliensis*. In order to drive expression it was necessary to include an intergenic region of the α-tubulin gene 5' to the *CAT* sequence (Laban and Wirth, 1989).

Stable extrachromosomal expression of *NEO* was also described in *L. enriettii*, with the *NEO* sequence flanked by α-tubulin intergenic sequences to process the *NEO* mRNA (Laban et al., 1990). *DHFR-TS* intergenic regions were also used to drive the expression of *NEO* from an ectopic plasmid to produce G418-resistant *L. major* (Kapler et al., 1990).

Intergenic regions of *Leishmania* genes were subsequently found to be essential to allow expression of active proteins from ectopic constructs. Expression of either *CAT* or *NEO* was only successful when the α-tubulin intergenic region, containing the trans-splicing site for the downstream gene, was included on the plasmid 5' to the *CAT* and *NEO* sequences (Curotto de Lafaille et al., 1992).

A series of expression vectors, named pNUS vectors, have been designed for the trypanosomatids *Crithidia* and *Leishmania*, allowing expression of introduced genes with His- or GFP-tags or signal sequences, for purification, visualisation or secretion of proteins (Tetaud et al., 2002). Proteins can also be expressed by these vectors with their native start and stop sites in order to obtain non-tagged proteins. The pNUS vectors contain an rRNA promoter, intergenic sequences from the *C. fasciculata* phosphoglycerate kinase locus both 5' and 3' to the introduced gene, and the 3' untranslated sequence of the *C. fasciculata* glutathionyl-spermidine synthetase locus 3' to the antibiotic resistance gene. A multiple cloning site allows for easy insertion of desired genes. Versions of the vectors are available containing three different antibiotic selection genes, *HYG*, *NEO* and bleomycin hydrolase (*BLE*) resistance genes.

5.1.3 Resistance markers

To genetically manipulate *Leishmania* a number of antibiotic resistance genes have been developed and are commonly used, with several markers generally necessary in order to manipulate genes in *Leishmania*. These genes are shown in Table 5.1.

Table 5.1 Antibiotic resistance genes used for genetic manipulation of *Leishmania*.

Gene	Encoded Protein	Antibiotic	Reference
NEO	Neomycin phosphotransferase	Geneticin or G418	Laban et al., 1990
SAT	Streptothricin acetyltransferase	Nourseothricin	Joshi et al., 1995
PAC	Puromycin N-acetyltransferase	Puromycin	Freedman and Beverley, 1993
HYG	Hygromycin B phosphotransferase	Hygromycin B	Lee and van der Ploeg, 1991
BLE	Bleomycin hydrolase	Phleomycin / Bleomycin	Souza et al., 1994
BSD	Blasticidin S deaminase	Blasticidin S	Brooks et al., 2000

5.1.4 Overview

Genetic manipulation in *Leishmania* is a well characterised method for establishing the importance of proteins. Utilisation of this method to generate null mutants and ectopic over-expression mutants of OPB in *L. major* will allow investigation of the role of OPB and the determination of its role in Bz-R-AMC cleavage. This will provide information on whether it is essential for parasite survival or is a potential drug target.

5.2 Results

5.2.1 Constructs for over-expression of OPB in *L. major*

In order to over-express OPB in *L. major*, the full length gene (LmF09.0770), was inserted into the HcN pNUS vector (Tetaud et al., 2002), with its start and stop sites intact, and transfected into *L. major*. As controls, two other constructs were transfected into *L. major*, one with the active site serine of OPB mutated to a glycine residue (OPB^{S577G}), and the other the empty pNUS vector.

The two constructs used for over-expression are shown in Figure 5.1. The full length gene was generated by PCR, using genomic *L. major* WT DNA as template, using the primers OL2352 and OL2353, ligated into the TOPO vector, and then transferred into the pNUS vector to give pGL1640. Site directed mutagenesis was then completed on pGL1640, using the primers OL2550 and OL2551 to mutate the active site serine (residue 577) to a glycine, giving the active site mutant construct, pGL1714. The empty pNUS vector, pGL1137, was not altered prior to use. Details of primers used are given in Section 2.5.3.

5.2.2 Over-expression of OPB

5.2.2.1 Generation of lines

Two populations of cells over-expressing OPB were produced, WT[*OPB*] populations 1 and 2, by splitting the transfected culture into two immediately following transfection and selecting using 50 µg/ml G418. A single population of cells was generated over-expressing OPB with the active site mutation, WT[OPB^{S577G}], by selection with 25 µg/ml G418. The cells were maintained in 50 µg/ml G418. A single population of cells with the empty pNUS vector was also produced, by selection with 50 µg/ml G418.

The ectopic constructs were shown to be present in the transfected populations by PCR analysis. A PCR product of 1.2 kb, amplified using the primers M13 Forward and OL2456, shows the construct is present in the WT[*OPB*] populations. A 1.7 kb PCR product, amplified using the primers M13 Reverse and OL816, shows the construct is present in the WT[OPB^{S577G}] and empty pNUS vector lines (Figure 5.2). In both, a product of the correct size was not found in the WT population.

A western immunoblot was carried out using anti-OPB antibody and anti-EF-1α antibody as a loading control. An increased level of the 83 kDa protein corresponding to OPB was detected in the two WT[*OPB*] lysates (lanes 2 and 3, Figure 5.3A), compared to the *L. major* WT lysate (lane 1, Figure 5.3A). There was also an increase of the OPB protein detected in the WT[OPB^{S577G}] lysate (lane 3, Figure 5.3B), with no increase in the level of protein expressed in the empty vector lysate (lane 2, Figure 5.3B), compared to the *L. major* WT lysate (lane 1, Figure 5.3B). In contrast, the same level of expression of the control protein, EF-1α, was found in all the lysates compared to *L. major* WT.

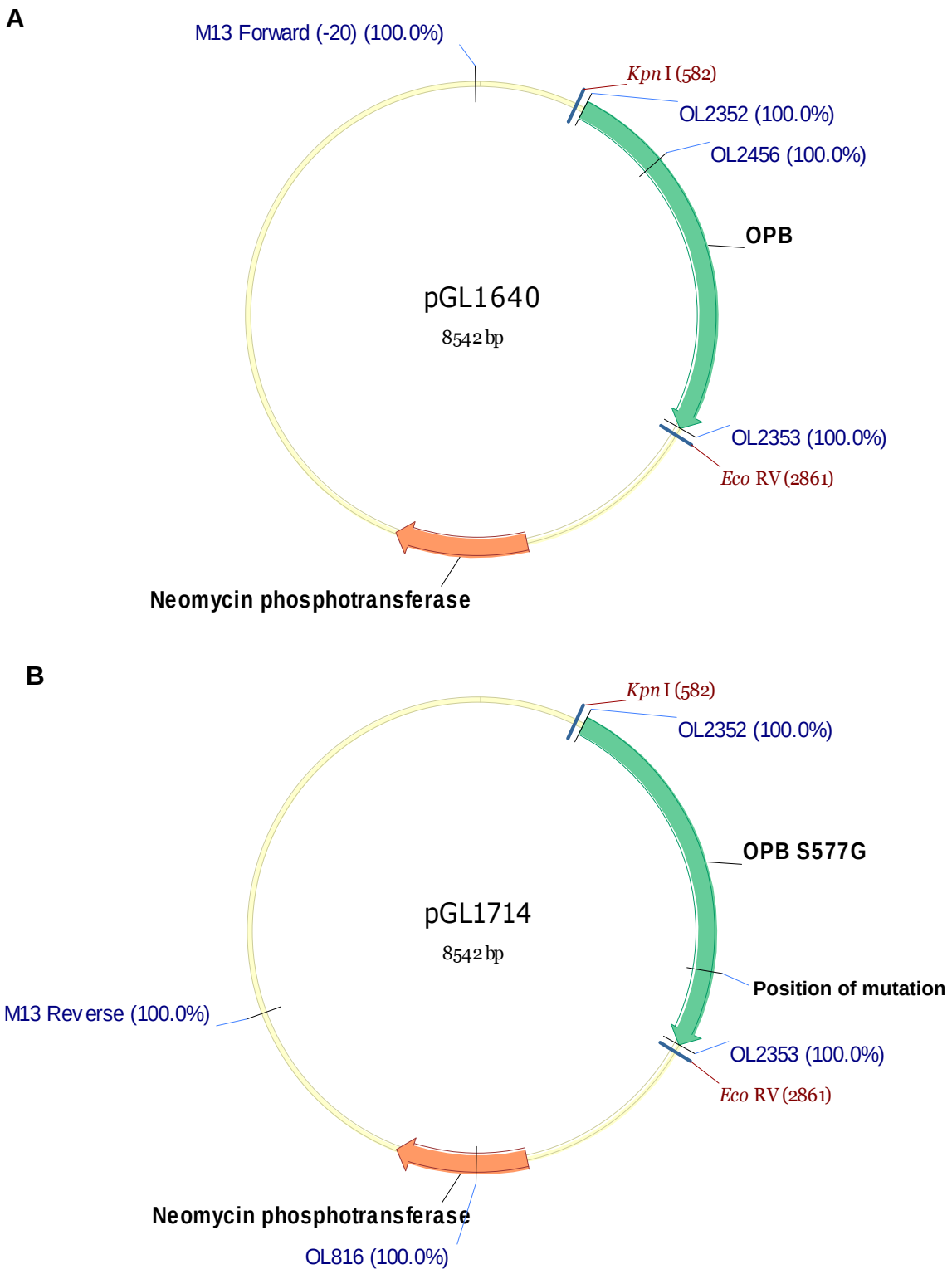


Figure 5.1 Constructs for the over-expression of OPB in *L. major*. A = OPB, B = OPB^{S557G}. In A, OPB denotes *L. major* OPB gene, LmF09.0770, and M13 Forward and OL2456 are the primers used to check for presence of the construct. In B, OPB S577G denotes *L. major* OPB with the serine residue at 577 mutated to glycine, and M13 Reverse and OL816 are the primers used to check for presence of the construct. In both, *Kpn*I and *Eco*RV are the restriction enzymes used to digest the *OPB* from the TOPO vector to insert it into the pNUS vector and OL2352 and OL2353 are the primers used to generate the gene by PCR.

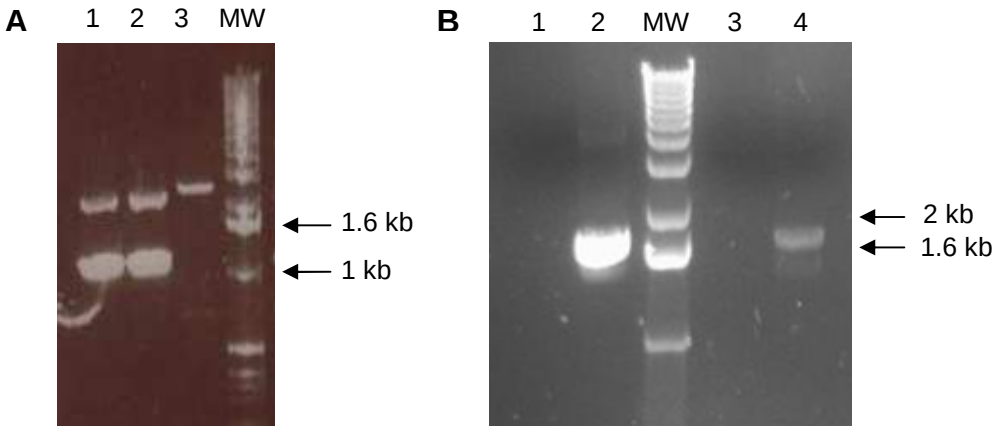


Figure 5.2 PCR analysis of OPB over-expressers. DNA was used from both over-expresser populations and *L. major* WT to determine presence of pGL1640 construct. A = PCR using genomic DNA from both WT OPB over-expressers. 1 = WT[*OPB*] population 1, 2 = WT[*OPB*] population 2, 3 = *L. major* WT and MW = 1 kb ladder. B = PCR using genomic DNA from active site mutant OPB over-expresser population and empty vector population, 1 and 3 = *L. major* WT, 2 = WT[OPB^{S577G}] population and 4 = empty vector population and MW = 1 kb ladder. The relevant size bands of the ladders are marked.

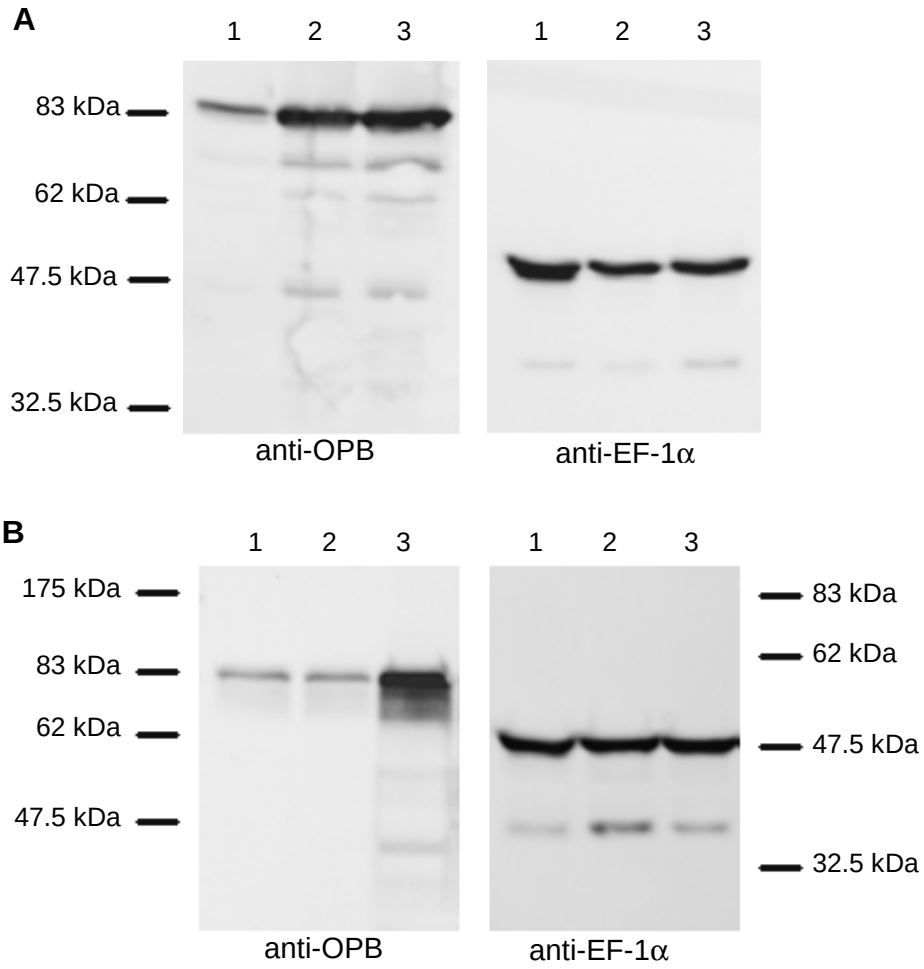


Figure 5.3 Demonstration of over-expression of OPB in *L. major* cells by western immunoblot. In A, 1 = *L. major* WT cells, 2 = WT[*OPB*] population 1 and 3 = WT[*OPB*] population 2. In B, 1 = *L. major* WT cells, 2 = empty pNUS vector population and 3 = WT[OPB^{S577G}] population. A lysate of 5 x 10^6 cells was run per lane, anti-OPB antibody was applied at a concentration of 1 in 20,000 and anti-EF-1α antibody was applied at a concentration of 1 in 10,000. HRP-conjugated anti-sheep and anti-mouse secondary antibodies were applied at a 1 in 5,000 concentration and the chemiluminescence was detected using a Molecular Imager ChemiDoc System.

5.2.2.2 Phenotype analysis

The growth of the WT[*OPB*] promastigote populations was compared to that of *L. major* WT, the WT[OPB^{S577G}] and the empty pNUS vector. The presence of the constructs in the promastigotes led to a slightly lower stationary phase population level being reached, but as this was the same for the OPB over-expressing lines and the empty vector it is likely that this is due to the antibiotic pressure in the culture. There was also an initial lag in the growth of the empty vector population, but this was not replicated in the OPB over-expressing lines. The rate of growth of the logarithmic phase was very similar for all the lines (Figure 5.4).

FACS analysis was performed to investigate the ploidy of the over-expresser lines. Over-expression of OPB had no effect on the ploidy of the *L. major* (Figure 5.5), as the cells all had normal proportions of 2N and 4N cells, representing normal cells and cells in the process of dividing, having already duplicated their DNA.

The level of peptidase activity towards Bz-R-AMC was determined in the various cell lines. The WT[*OPB*] promastigotes had over 20 times the enzymatic activity of the wild type cells or the empty vector control. The WT[OPB^{S577G}] promastigotes had an activity that was approximately half that of the wild-type (Figure 5.6).

The effect of over-expressing OPB in *L. major* on the IC_{50} value for antipain, leupeptin and pefabloc inhibition of peptidase activity towards Bz-R-AMC was measured. Over-expression of OPB caused a small increase in the IC_{50} for antipain compared to both *L. major* WT and empty vector promastigotes, whilst the IC_{50} for pefabloc was only higher for the WT[*OPB*] populations compared to *L. major* WT promastigotes, though only the difference between the *L. major* WT and the WT[*OPB*] population 2 was significant, with a p value of 0.04 by an unpaired t-test. There was no difference between the IC_{50} values of leupeptin for any of the lines (Table 5.2).

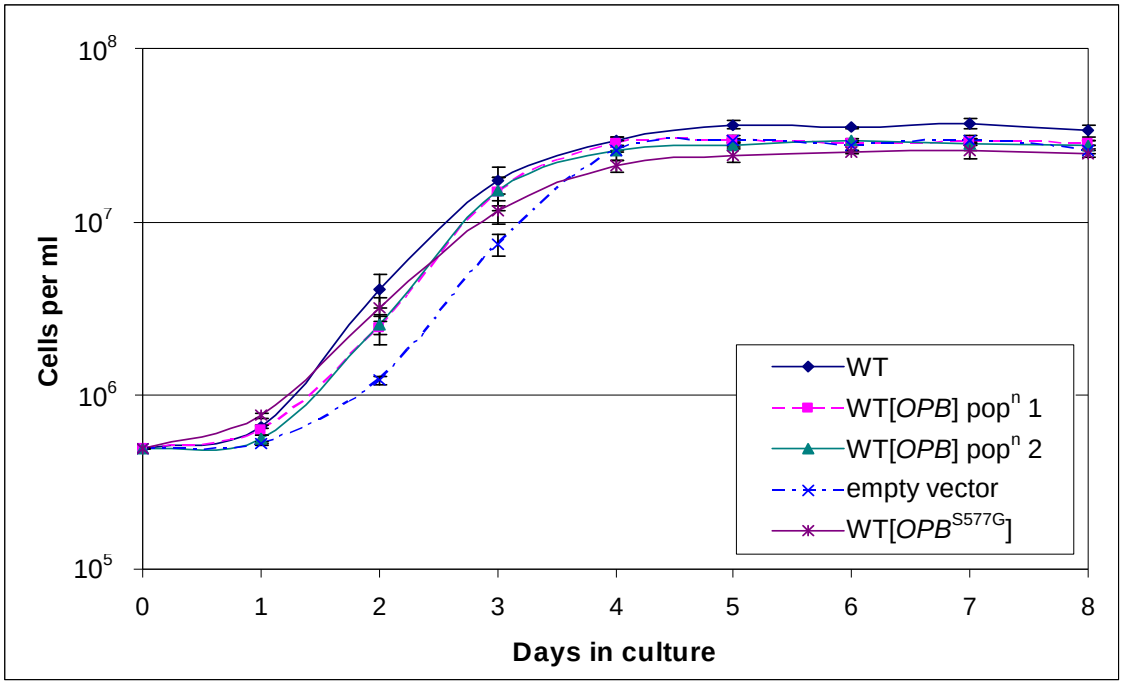


Figure 5.4 Effect of over-expression of OPB on growth of *L. major* promastigotes. Cells were counted over 8 days in culture. Three cultures were counted of each line to generate a mean, with values ± SEM.

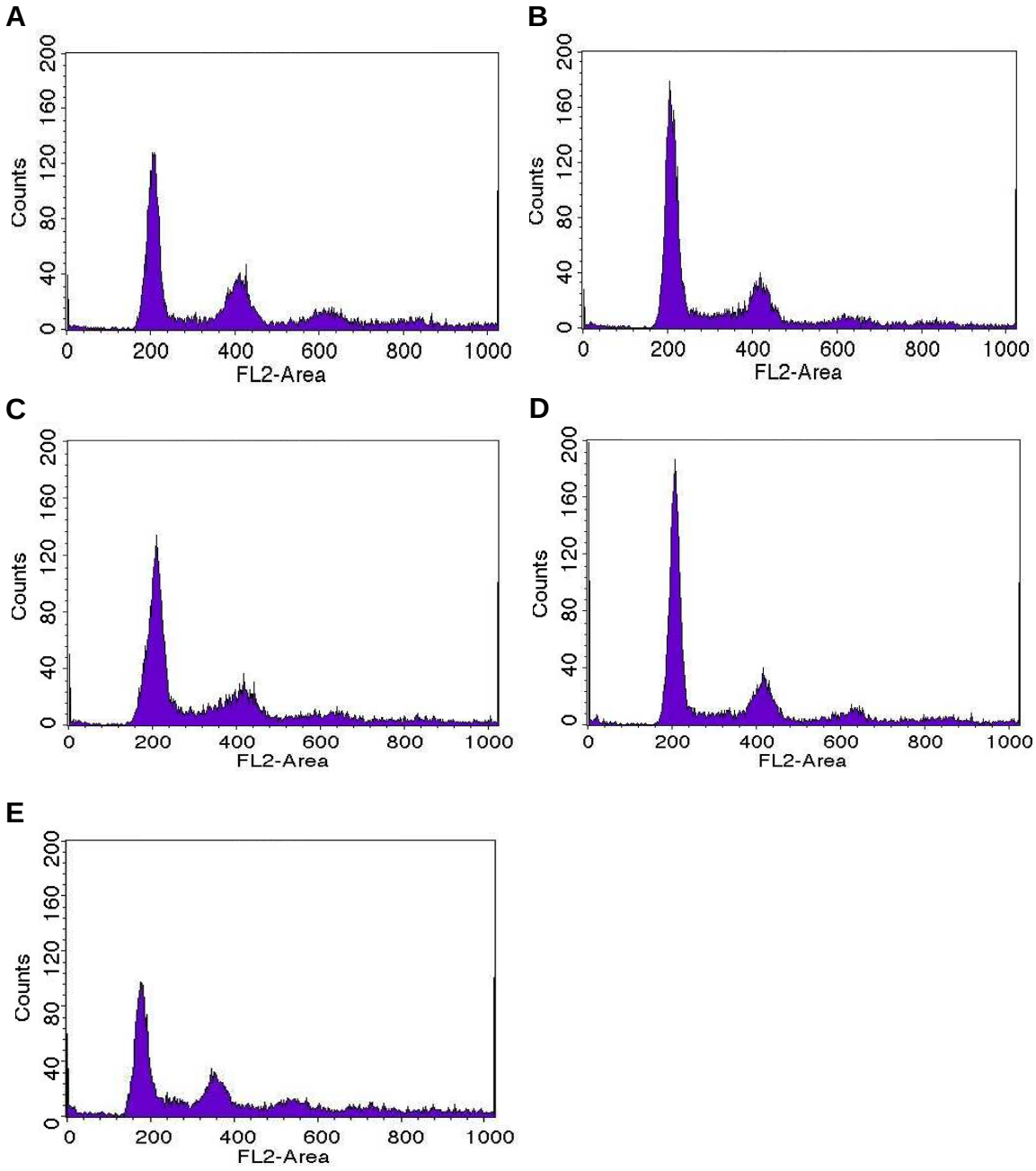


Figure 5.5 Effect of over-expression of OPB on ploidy of promastigotes. FACS analysis was performed on *L. major* WT and promastigotes containing pNUS empty vector and OPB over-expressing populations. A = *L. major* WT, B = pNUS empty vector, C = WT[*OPB*] population 1, D = WT[*OPB*] population 2 and E = WT[OPB^{S577G}]. Peaks at 200 FL2-Area represent 2N cells and peaks at 400 FL2-Area represent 4N cells.

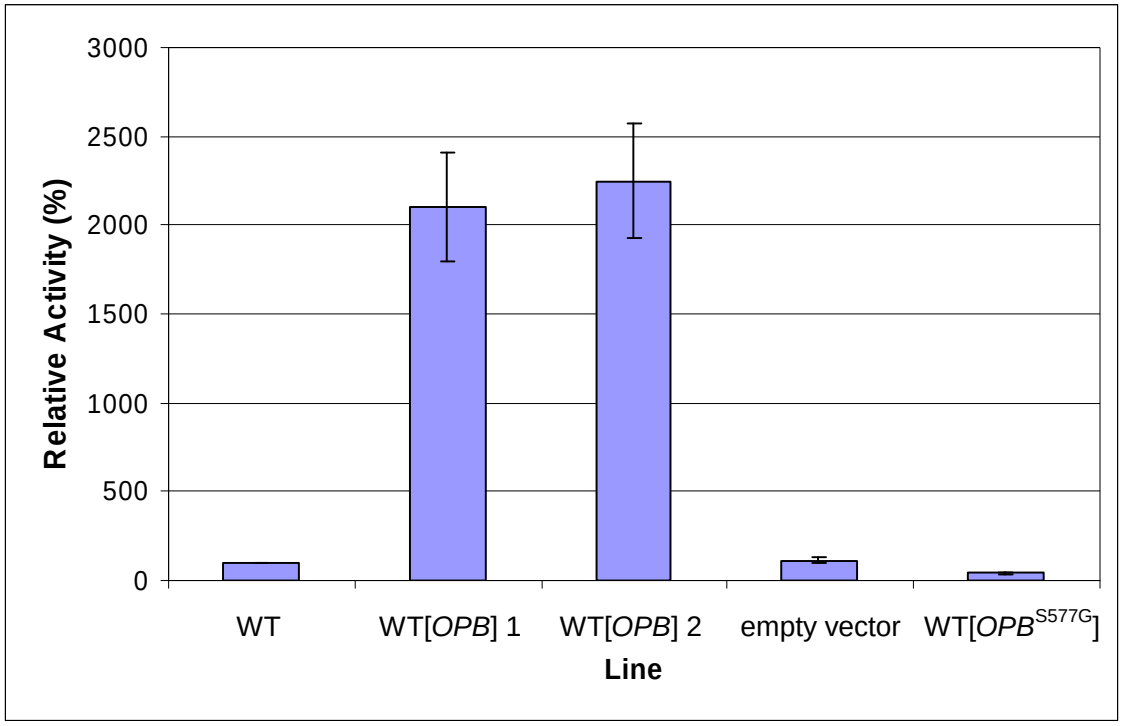


Figure 5.6 Effect of over-expression of OPB in *L. major* promastigotes on the cleavage of 5 µM Bz-R-AMC. Over-expressers were compared to wild-type (taken as 100%) and empty vector control promastigotes. For each assay, 6 x 10^6 cells were used. Relative activity was calculated compared to WT for each individual experiment. The mean of between 4 and 11 experiments is shown ± SEM.

Table 5.2 Effect of over-expression of OPB in *L. major* promastigotes on the mean IC_{50} value for inhibition of cleavage of Bz-R-AMC, compared to wild-type and empty vector cells.

	L. major WT	WT[*OPB*] pop^n 1	WT[*OPB*] pop^n 2	empty pNUS vector
Mean antipain IC_{50} ± SEM (nM)	9.3 ± 1.6	23.6 ± 9.2	18.1 ± 5.9	12.5 ± 3.7
Mean leupeptin IC_{50} ± SEM (nM)	48.1 ± 5.8	57.2 ± 14.4	53.3 ± 8.3	55.9 ± 7.3
Mean pefabloc IC_{50} ± SEM (µM)	14.4 ± 0.6	25.5 ± 6.4	25.4 ± 2.3	20.9 ± 3.9

Antipain has previously been shown to have anti-leishmanial activity (Coombs et al., 1982; Coombs and Baxter, 1984). As an inhibitor of OPB, it was hypothesised that OPB could be its intracellular target. Therefore, the effect of over-expression of OPB on the LD_{50} of antipain against promastigotes was analysed. A serially diluted drug assay was completed and the growth of parasites analysed by the reduction of alamar blue, to give the LD_{50} values. A slightly higher LD_{50} was found in the OPB over-expressing lines when compared to the wild-type (Figure 5.7), though only the increase in LD_{50} for the WT[*OPB*] population 2 compared to wild-type was significant, with a p value of 0.05, by an unpaired t-test.

5.2.3 Constructs for generating *OPB* null mutants in *L. major*

The constructs used for deleting the *OPB* gene in *L. major* are shown in Figure 5.8 and a schematic of the *OPB* locus and the constructs is shown in Figure 5.9. The 5' flanking region of *OPB* was generated by PCR using the primers OL2395 and OL2415, giving a flank of 872 bp and the 3' flanking region of *OPB* was generated by PCR using the primers OL2356 and OL2459, giving a flank of 539 bp. Details of primers used are given in Section 2.5.3. The 5' flank was ligated into the PCR-Script cloning vector and the 3' flank was ligated into the TOPO cloning vector, then both were inserted into the knock out construct that had previously been used to generate the *CPB* array and *ICP* null mutants (Mottram et al., 1996b; Besteiro et al., 2004), to give pGL1693. The *HYG* gene in pGL1693 was then replaced with the streptothricin acetyltransferase (*SAT*) gene to give pGL1792. The linear null cassette was excised using the restriction enzyme *Bgl* II before transfection into *L. major*. pGL1693 was used for the first round of transfection, with pGL1762 used for the second round.

5.2.4 *OPB* null mutants

5.2.4.1 Generation of lines

Two independent populations of *OPB* heterozygote cells were generated by splitting the transfected culture into two immediately following transfection. Prior to attempting to knock out the second allele of *OPB* it was necessary to first demonstrate that the first allele had been removed.

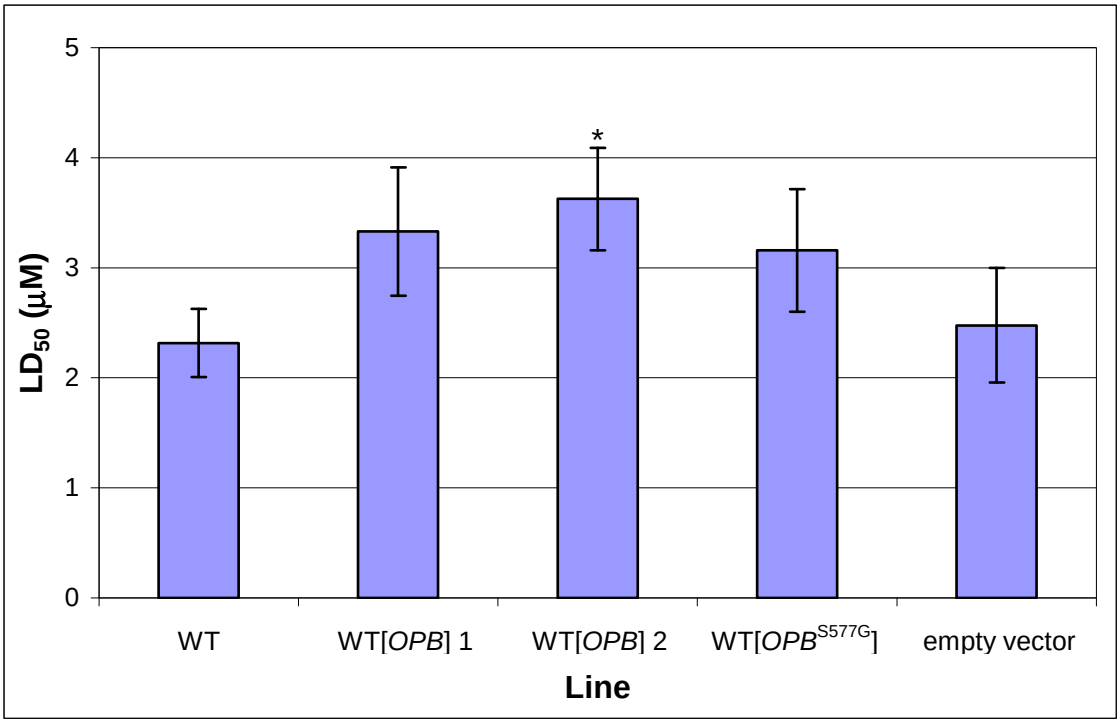
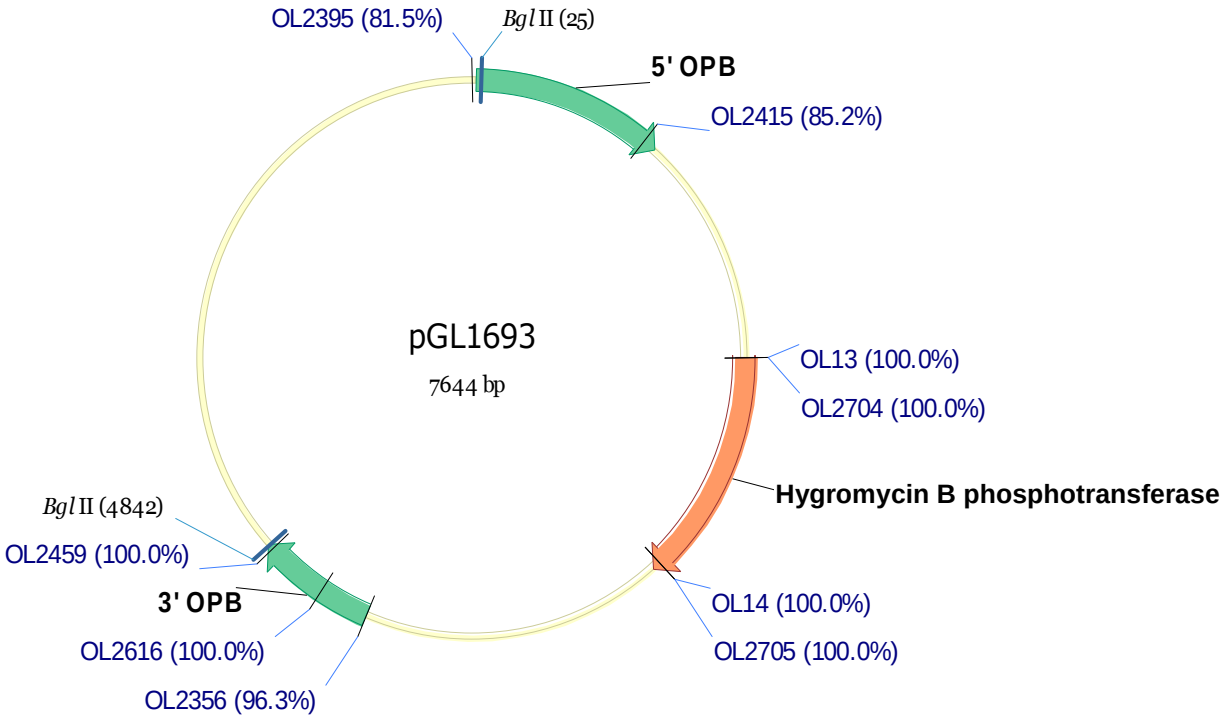


Figure 5.7 LD_{50} of antipain against OPB over-expressing promastigotes. LD_{50} values were determined from 6 day serial dilution assays on promastigotes, detected by reduction of alamar blue. The mean of between 4 and 8 experiments is shown ± SEM. * = p = 0.05, compared to *L. major* WT, by an unpaired t-test.

A



B

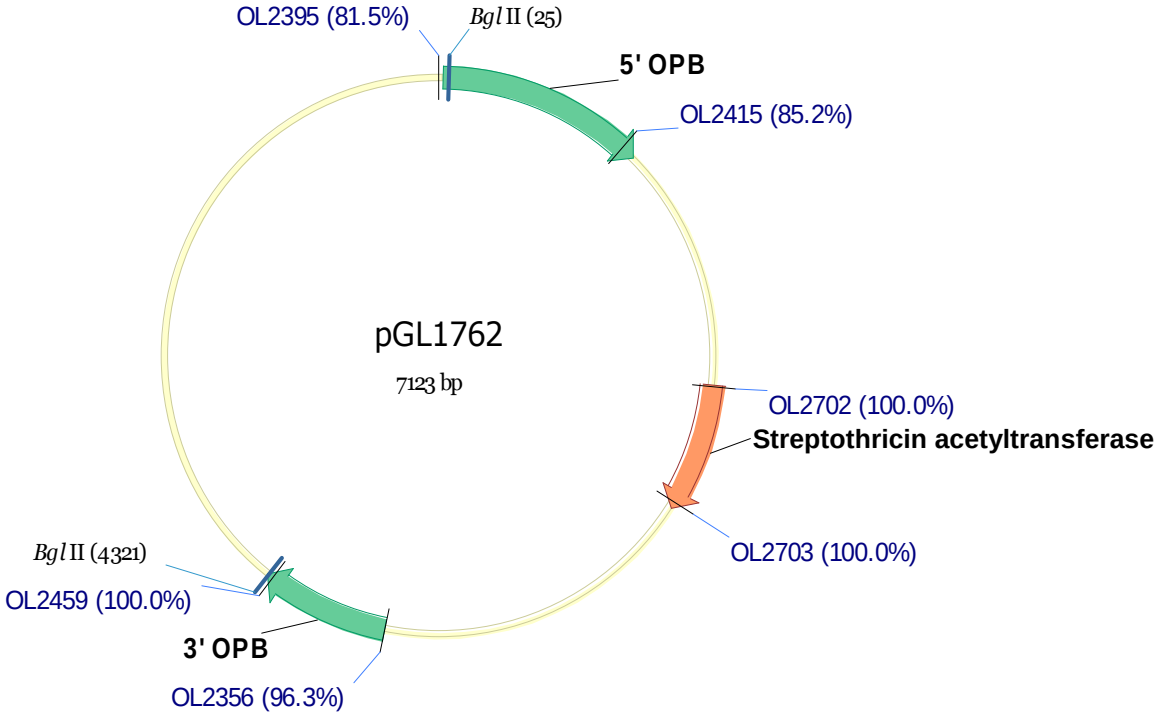


Figure 5.8 Construct for the knock out of *OPB* in *L. major*. A = pGL1693, B = pGL1762. 5' *OPB* denotes the 5' flanking region of the *L. major OPB* gene, 3' *OPB* denotes the 3' flanking region of the *L. major OPB* gene, that were used. The primers used for PCR reactions (OL2616, OL14 & OL13; OL2704 & OL2705 and OL2702 &OL2703), to check for integration and presence of the construct, as described in Sections 5.2.3 and 5.2.4.1, are shown. *Bgl* II indicates the restriction enzyme used to digest the construct prior to transfection into *L. major*.

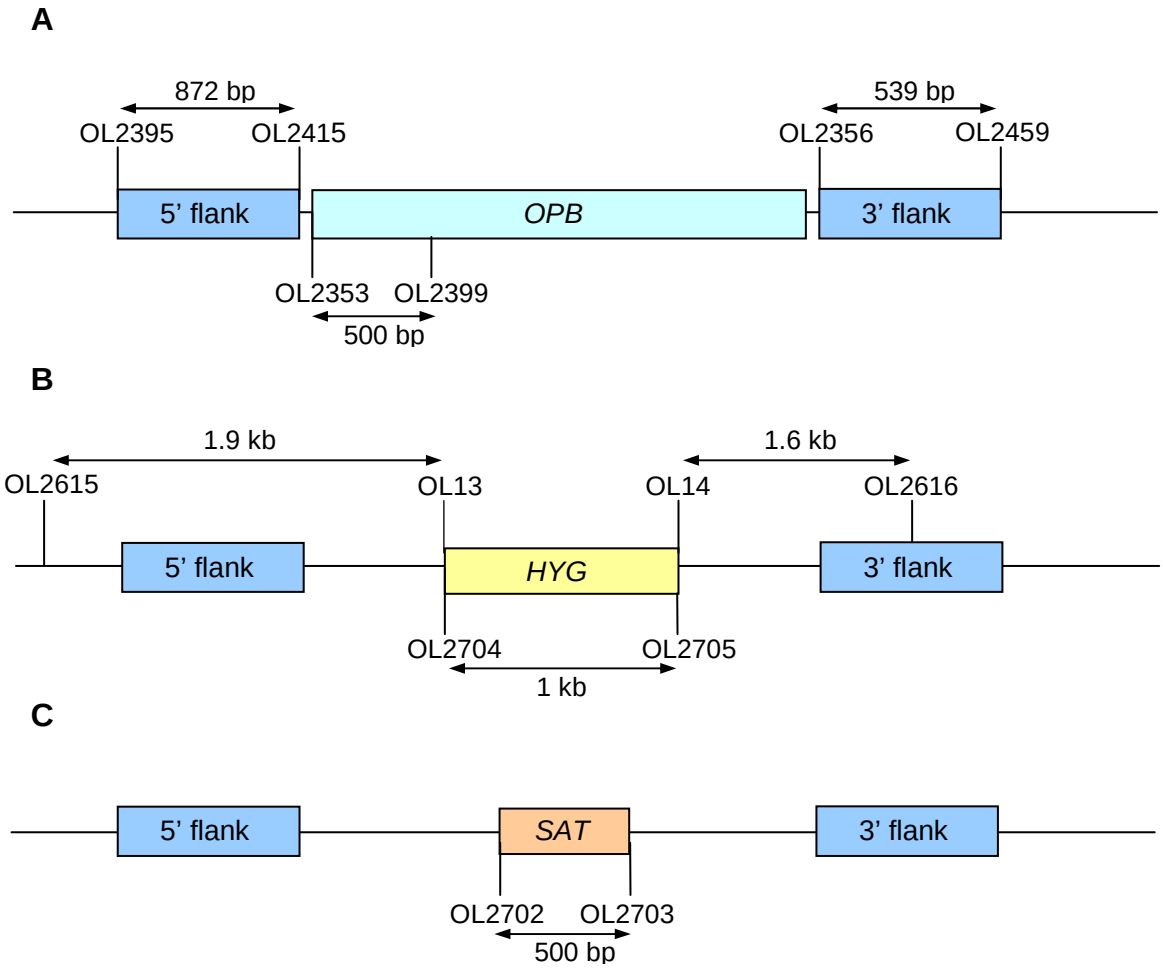


Figure 5.9 Schematic representation of *OPB* locus and plasmid constructs used for gene replacement. A = *OPB* locus, B = pGL1693 and C = pGL1762. Positions of primers used for PCR reactions and the length of PCR products produced, as described in Sections 5.2.3 and 5.2.4.1, are shown.

PCR analysis was carried out using two sets of primers. One set amplified a section of the construct from the hygromycin resistance gene to the 3' flank, showing that the construct was present. The second set amplified a section from the hygromycin resistance gene to outside the 5' flank, which would only generate a PCR product when the construct had correctly integrated into the genome. Both PCR products were only found with the DNA from the heterozygote populations and not with that from the *L. major* WT promastigotes (Figure 5.10), with both the heterozygote populations had the 1.9 kb PCR product amplified by primers OL2615 and OL13 indicating integration of the deletion construct and the 1.6 kb PCR product amplified by primers OL2616 and OL14 indicating presence of the deletion construct.

Transfection with *Bgl* II digested pGL1762 was carried out on heterozygote population 1. Immediately following transfection the culture was split into two, to ensure that if clones were generated from both cultures then they would be from independent transfection events. The two cultures were serially diluted and plated out on 96-well plates to generate clones (as described in Section 2.5.7.2). Following selection with hygromycin and nourseothricin, 33 clones were generated, which were analysed to determine if they were null mutant clones.

A primary screen was conducted to determine if the WT *OPB* locus was present in the clones using PCR. Clones that were negative were then checked for the presence of the antibiotic selection genes, also by PCR. The analysis was completed using three sets of primers. One pair, OL2353 and OL2399, amplified a 1 kb section of the WT *OPB* locus, the second pair, OL2702 and OL2703, amplified the *SAT* resistance gene, and the third pair, OL2704 and OL2705, amplified the *HYG* resistance gene. In *OPB* null mutants the WT locus should be missing and both the antibiotic genes should be present, with the opposite being true for WT cells (Figure 5.11). By this method only two clones were found to be potential null mutants: clones 10 and 21. These were the only two clones found to lack the 1 kb PCR product indicating presence of the WT locus. The PCR products amplified by clones 11 and 20 are included to illustrate the presence of the WT locus in these clones, which was replicated in the remaining 29 clones generated by the serial dilution. Both the 500 bp fragment amplified using primers for *SAT* and the 1 kb fragment amplified using primers for *HYG* were also found in clones 10 and 21. These clones were named Δ*opb* clone 10 and Δ*opb* clone 21.

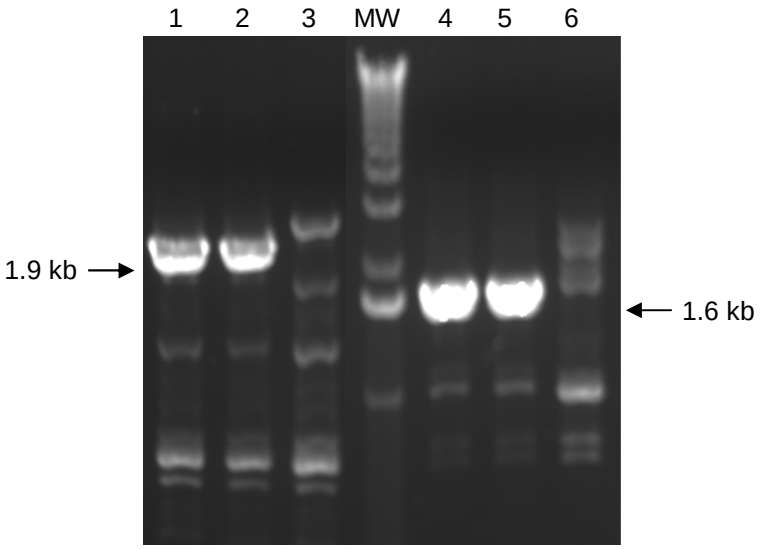


Figure 5.10 PCR to determine presence and integration of digested pGL1693. For lanes 1-3, a PCR product of 1.9 kb indicates that pGL1693 has been integrated, for lanes 4-6 a PCR product of 1.6 kb indicates that pGL1693 is present. Lanes 1 & 4 = heterozygote population 1; 2 & 5 = heterozygote population 2; 3 & 6 = *L. major* WT. MW = 1 kb ladder.

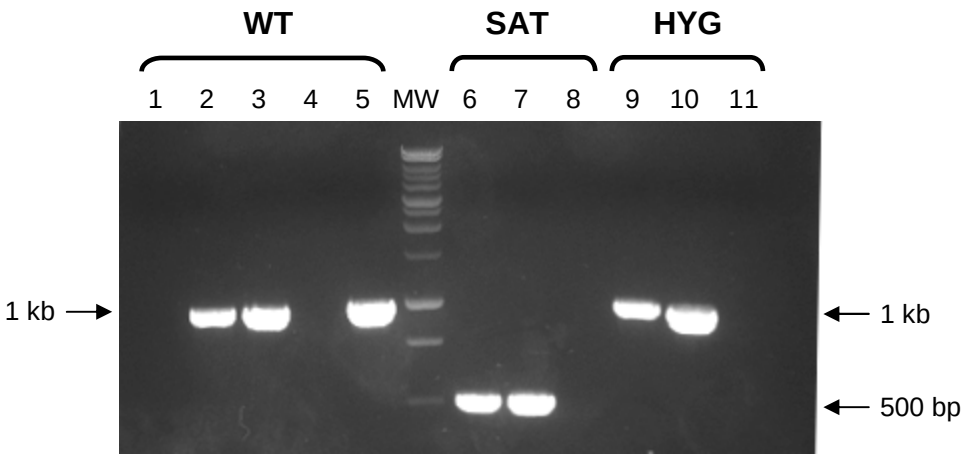


Figure 5.11 PCR to determine presence of WT *OPB*, *SAT* and *HYG*. DNA from potential null mutant clones and *L. major* WT promastigotes was used. For lanes 1-5 a PCR product of 1 kb indicates presence of the WT *OPB* locus, for lanes 6-8 a PCR product of 500 bp indicates presence of the *SAT* gene and for lanes 9-11 a PCR product of 1 kb indicates presence of the *HYG* gene. Lanes 1, 6 & 9 = clone 10; 2 = clone 11; 3 = clone 20; 4, 7 & 10 = clone 21; 5, 8 & 11= *L. major* WT. MW = 1 kb ladder.

A western immunoblot was carried out using anti-OPB antibody and anti-EF-1α antibody as a loading control. The 83 kDa protein corresponding to OPB was detected in lane 1, the WT *L. major* lysate, but there was no protein detected in either of the *OPB* null mutant clone lysates (lanes 2 and 3). In contrast, the same level of expression of the control protein, EF-1α, was found in all three lysates (Figure 5.12).

5.2.4.2 Phenotype analysis

The growth of the two Δ*opb* clones was compared to that of *L. major* WT promastigotes. The clones had a slight growth defect, only reaching a stationary phase concentration of less than 3×10^7 cells/ml, whilst the WT cells reached approximately 3.5×10^7 cells/ml. The clones also exhibited an initial lag in their growth and have a growth rate in log phase 20% slower than that of the wild-type (Figure 5.13).

To investigate the ploidy of the *OPB* null mutants, FACS analysis was performed, with the lines all found to have normal proportions of 2N and 4N promastigotes (Figure 5.14).

Since OPB had been shown to be responsible for the majority of Bz-R-AMC cleavage activity in *L. major*, the activity for the heterozygote and Δ*opb* clones was investigated. The rate of cleavage was determined for each line of promastigotes, as described in Section 2.4.1. The rates for the heterozygote and null mutant clones were then calculated as a percentage of the rate of cleavage of the *L. major* WT. The heterozygote was found to have around 42% of the cleavage compared to the WT whilst the Δ*opb* clones only had between 1.4 and 0.7% of the cleavage of the WT (Figure 5.15).

Since antipain is an inhibitor of OPB, it was hypothesised that OPB could be its intracellular target. Therefore, the LD_{50} of antipain for heterozygote and *OPB* null mutant clones was compared to that of *L. major* WT promastigotes. A serially diluted drug assay was completed and the growth of parasites was analysed by the reduction of alamar blue, to give the LD_{50} values. The OPB heterozygote population 1 showed a small increase in sensitivity to antipain, however the decrease in the LD_{50} was not significant (p value of 0.14, by an unpaired t-test). There was a significant decrease in the LD_{50} for Δ*opb* clone 21

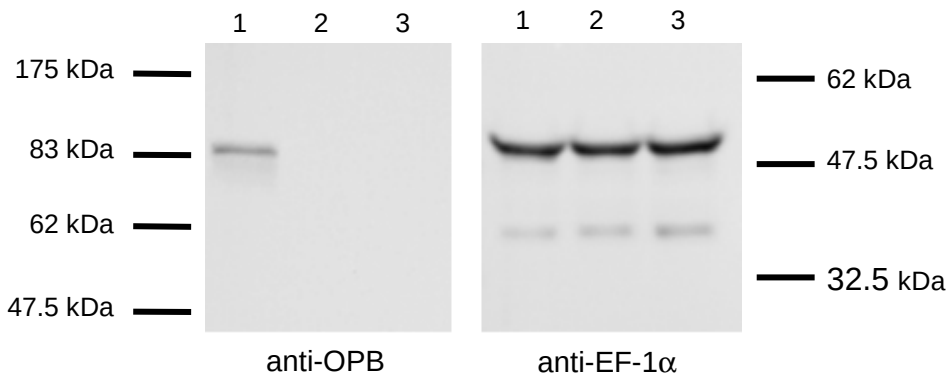


Figure 5.12 Demonstration of OPB protein levels in *L. major* WT promastigotes and Δ*opb* by western immunoblot. 1 = *L. major* WT cells, 2 = Δ*opb* clone 10 and 3 = Δ*opb* clone 21. A lysate of 5 x 10^6 cells was run per lane, anti-OPB antibody was applied at a concentration of 1 in 20,000 and anti-EF-1α antibody was applied at a concentration of 1 in 10,000. HRP-conjugated anti-sheep and anti-mouse secondary antibodies were applied at a concentration of 1 in 5,000 and the chemiluminescence was detected using a Molecular Imager ChemiDoc System.

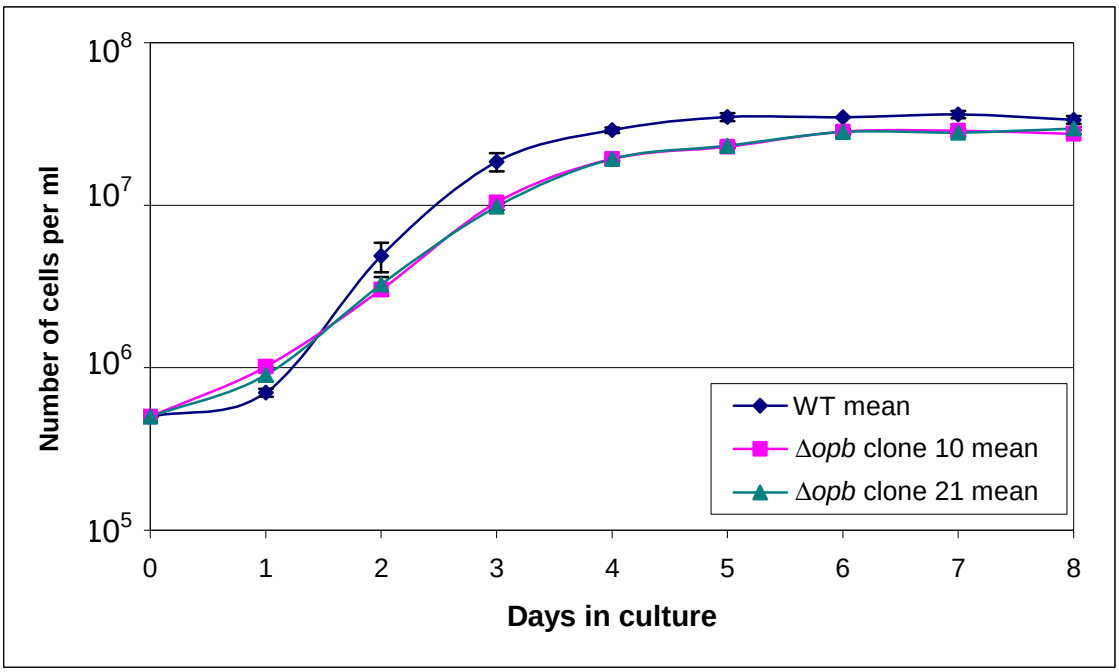


Figure 5.13 Effect of deleting *OPB* in *L. major* cells on growth of promastigotes. Cells were counted over 8 days in culture. No antibiotics were applied to the Δ*opb* clones. Three cultures were counted of each line to generate a mean ± SEM.

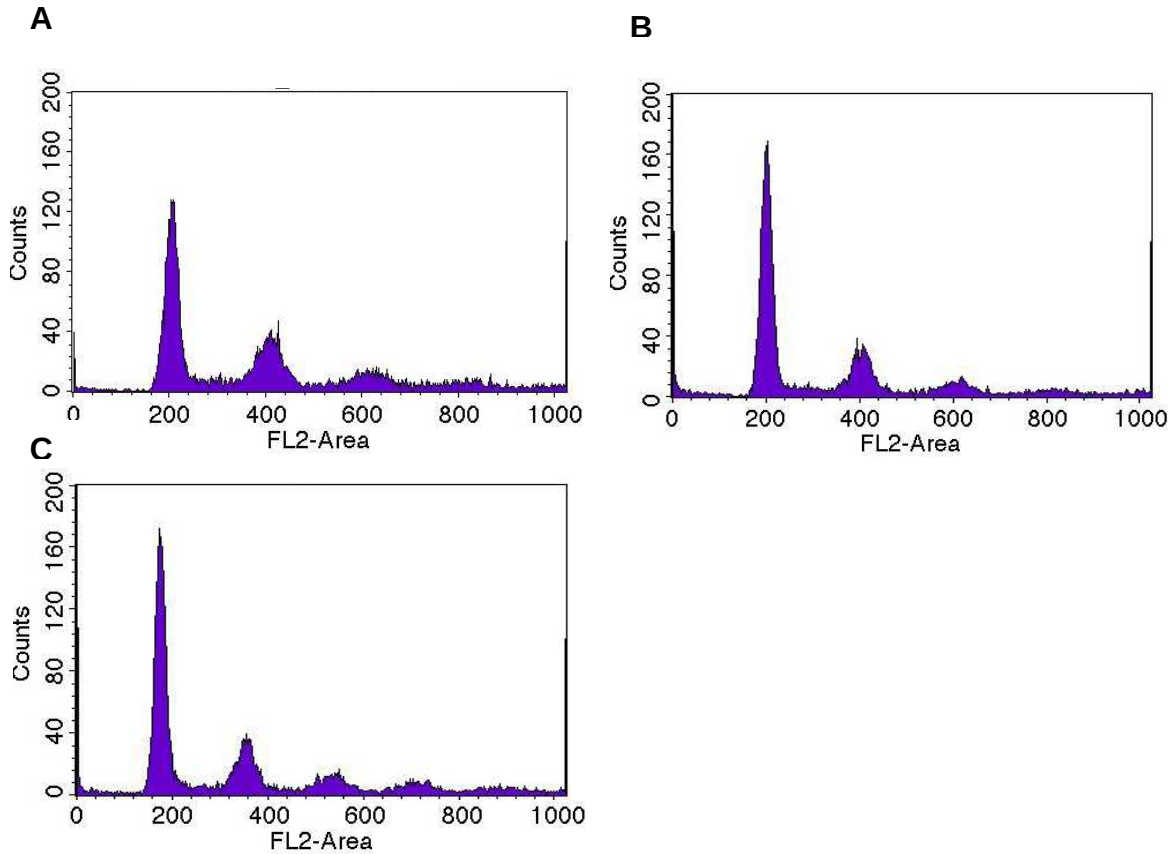


Figure 5.14 Comparison of FACS profiles of WT *L. major* and the two Δ*opb* clones. A = *L. major* WT, B = Δ*opb* clone 10, C = Δ*opb* clone 21. Peaks at 200 FL2-Area represent 2N cells and peaks at 400 FL2-Area represent 4N cells.

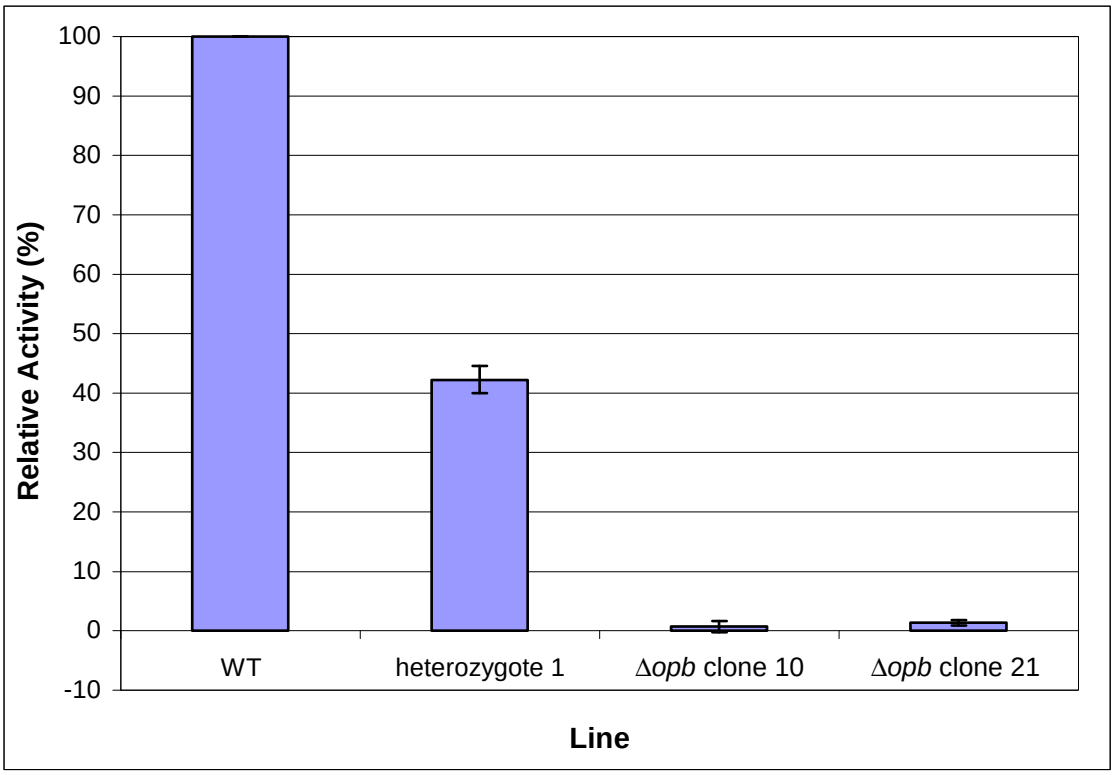


Figure 5.15 Cleavage of 5 μM Bz-R-AMC. Relative activities of live heterozygote and Δ*opb* clone promastigotes as a percentage of *L. major* WT promastigotes were determined from the rate of increase in fluorescence. Values are the mean of between 5 and 11 experiments, ± SEM.

compared to *L. major* WT, with a p value of 0.02 in an unpaired t-test. However, the LD_{50} of Δ*opb* clone 10 was not decreased and was the same as that of *L. major* WT (Figure 5.16).

Δ*opb* and *L. major* WT stationary phase cells were used to infect peritoneal exudate macrophages (PEM) at an 8:1 ratio of parasites to macrophages. PEM were fixed after one, four and six days post infection and the numbers of infected macrophages were counted (Figure 5.17). After one day both WT and Δ*opb* cells infected PEM at a similar level, between 60-75%. However, by day six only 5-10% of PEM were still infected with Δ*opb* promastigotes, whereas over 80% were infected with *L. major* WT promastigotes. The infection levels were significantly lower at day 6 for the null mutant clones compared to *L. major* WT, with p values of 0.001 (clone 10) and 0.002 (clone 21) by an unpaired t-test.

A reduced ability to survive infection in macrophages can reflect a reduced number of metacyclic cells. The number of metacyclic promastigotes in Δ*opb* clones was compared to that of *L. major* WT. Two methods were used, i) western immunoblotting for the surface protein HASPB (Figure 5.18), a known metacyclic marker (Flinn et al., 1994), and ii) comparison of peanut lectin agglutination of cells (Table 5.3), which agglutinates the non-metacyclic cells (Sacks et al., 1985). Immunoblotting of lysates of *L. major* WT and *OPB* null mutant clones stationary phase promastigotes determined that there was less HASPB protein, consistent with there being fewer metacyclic promastigotes in the two null mutant clones, but no reduction in the level of the control protein, EF-1α (Figure 5.18). The reduction in metacyclic promastigotes was replicated by the peanut lectin agglutination of the lines, with a reduction in the apparent number of metacyclics of 30% for clone 10 compared to the wild-type and 64% for clone 21 (Table 5.3).

Table 5.3 Comparison of percentages of metacyclic promastigotes in *L. major* WT and Δ*opb* clones as determined by peanut lectin agglutination.

Line	% Metacyclics
L. major WT	35.1 ± 0.2
Δ*opb* clone 10	24.6 ± 2.4
Δ*opb* clone 21	12.6 ± 0.1

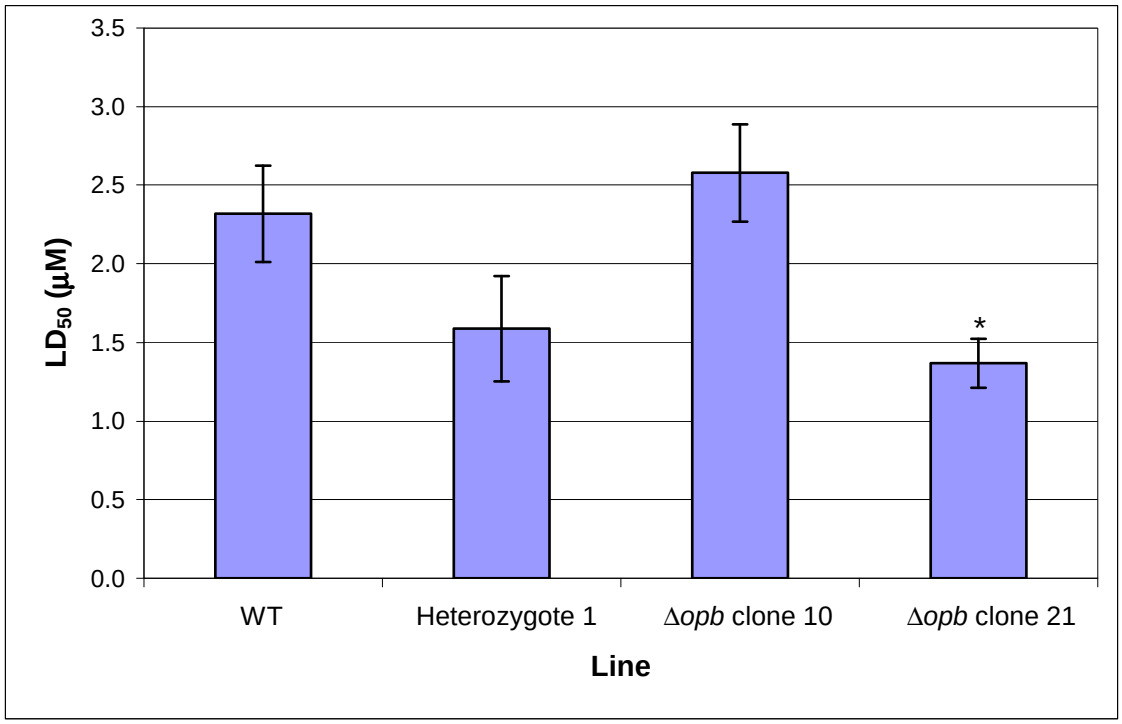


Figure 5.16 LD_{50} of antipain against heterozygote and Δ*opb* promastigotes. LD_{50} values were determined from 6 day serial dilution assays on promastigotes, detected by reduction of alamar blue. The mean of between 5 and 8 experiments is shown ± SEM. *** =** p = 0.02, compared to *L. major* WT, by an unpaired t-test.

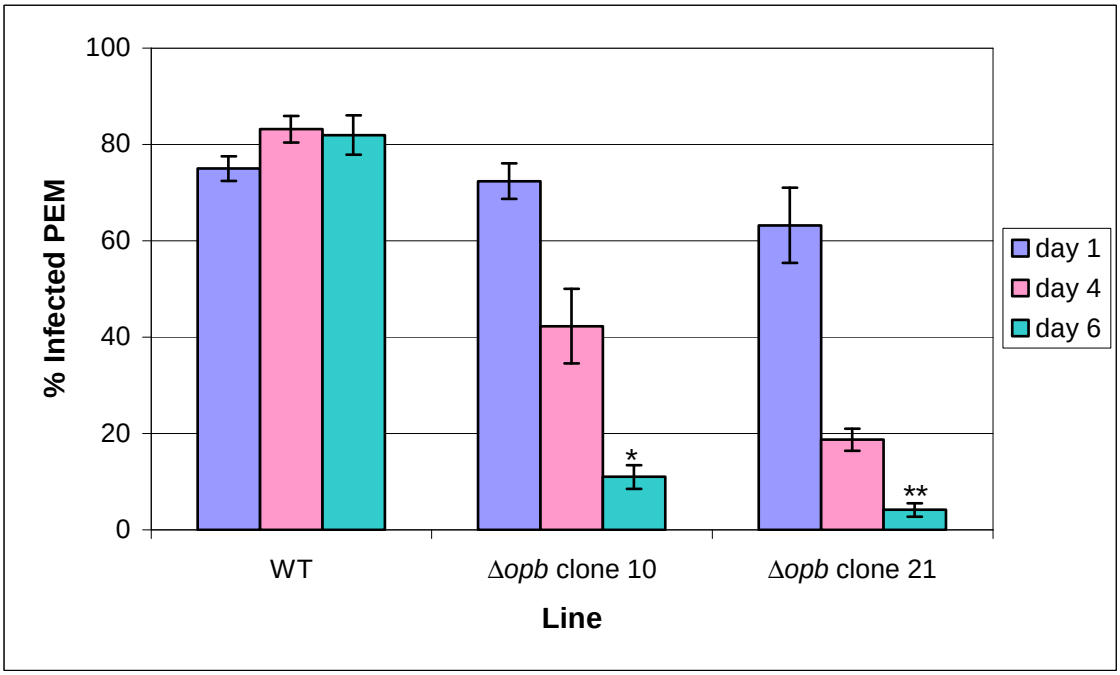


Figure 5.17 Infectivity of Δ*opb* clones compared to WT *L. major* in PEM. PEM were infected at a ratio of 8 stationary phase promastigotes to 1 macrophage and were incubated for 6 days. Each value is the mean of 5-8 replicates over two experiments ± SEM. * = p = 0.001 and ** = p= 0.002, compared to *L. major* WT at day 6, by unpaired t-test.

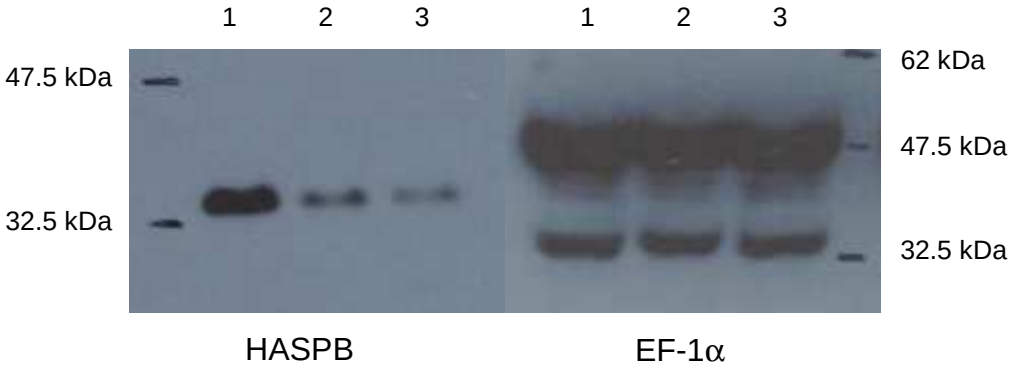


Figure 5.18 Western immunoblot of Δ*opb* clones and *L. major* WT to compare HASPB levels. 1 = *L. major* WT cells, 2 = Δ*opb* clone 10 and 3 = Δ*opb* clone 21. A lysate of 5 x 10^6 cells was run per lane, anti-HASPB antibody was applied at a concentration of 1 in 5,000 and anti-EF-1α antibody was applied at a concentration of 1 in 10,000. HRP-conjugated secondary antibodies were applied at a concentration of 1 in 5,000 and the chemiluminescence was detected in an Xomat processor.

To assess if the null mutant clones were infective to mice and could generate lesions, Δ*opb* and *L. major* WT stationary phase promastigotes were used to inoculate BALB/c mice footpads. Footpads were measured at weekly intervals and mice were culled once the footpad diameter was 5 mm. There was a lag in the growth of the footpads in mice inoculated with the Δ*opb* clones compared to those inoculated with *L. major* WT. There was also a slower rate of growth in the lesions of Δ*opb* once the exponential rate of growth started at week 3 for the clones, compared to week 1 for *L. major* WT. Δ*opb* clone 21 had a slower rate of lesion development compared to Δ*opb* clone 10 (Figure 5.19).

5.3 Discussion

The pNUS vector had been used previously within the Mottram laboratory to over-express the *L. major* proteins VPS4, ATG4.2 and metacaspase (Besteiro et al., 2006; Ambit et al., 2008) and was thus chosen for the over-expression of OPB. Over-expression of OPB was successfully achieved in *L. major* promastigotes, as shown by increased level of protein (Figure 5.3) and the higher rate of cleavage of Bz-R-AMC (Figure 5.6). This confirmed that the higher level of OPB protein found by western immunoblotting was responsible for faster cleavage of Bz-R-AMC. The increase in protein level found by western blot was not quantified, but a large increase is clear. Over-expression of OPB^{S577G} was also confirmed by western immunoblot (Figure 5.6), but over-expression of the mutant led to a drop in activity relative to wild-type promastigotes, suggesting that the active site mutant had a dominant negative effect. It may be that this is due to the OPB^{S577G} binding the substrate but being unable to cleave it, acting in a similar manner to a competitive inhibitor.

It has been previously shown that non-native expression levels of proteins can be deleterious, as with the metacaspase cysteine peptidase. For metacaspase (MCA), removal of even the first allele of the peptidase was impossible without expression of a *MCA* transgene elsewhere in the parasite's genome, thus maintaining a native level of expression, and over-expression of MCA at higher than normal levels led to aneuploid cells with a severe growth defect (Ambit et al., 2008). The level of expression of OPB is not critical for *L. major* as the over-expression of either the WT or the active site mutant OPB in promastigotes had no

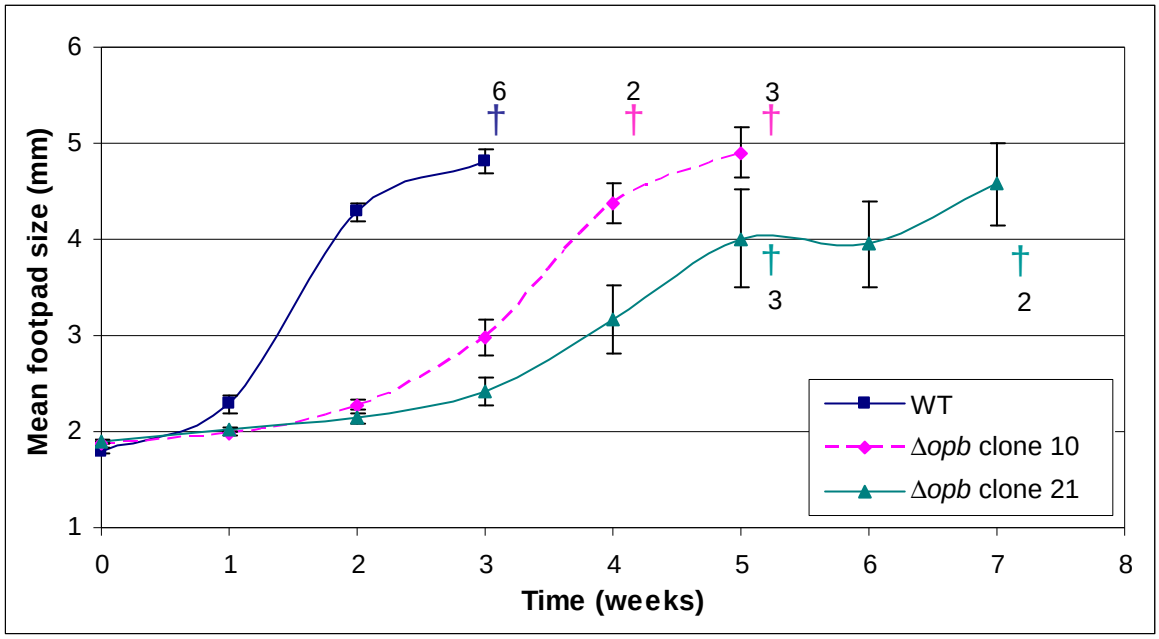


Figure 5.19 Growth of footpad lesions of BALB/c mice. Footpads infected with Δ*opb* clones were compared to footpads infected with WT *L. major*. 5 x 10^5 stationary phase promastigotes were injected per footpad. Each value is the mean of 5 or 6 mice ± SEM. Crosses and numbers represent the number of mice culled at that number of weeks.

major growth defect compared to the empty vector control line (Figure 5.4) and there was no effect on their ploidy (Figure 5.5).

As OPB activity against Bz-R-AMC was reduced by the inhibitors antipain, leupeptin and pefabloc, it was hypothesised that the presence of higher levels of OPB would lead to the IC_{50} values for inhibition increasing. The IC_{50} values for antipain and pefabloc for the over-expresser lines did increase relative to the wild-type promastigotes, but not relative to the empty vector line (Table 5.2). This suggests that it was the presence of the ectopic construct itself that was somehow causing the alteration in the IC_{50} values. There was only approximately a 100% and a 50% increase in the IC_{50} values for antipain and pefabloc respectively, which is a very low difference in the values compared to the over 20-fold increase in the cleavage rate. There was no change in the values for leupeptin. However, an IC_{50} is independent of enzyme concentration. The IC_{50} is based on a ratio of the rate of a reaction, which is itself derived from the Michaelis-Menten equation Enzyme concentration is not a parameter in the Michalis-Menton equation as long as all other reaction components are in excess, meaning the concentration of enzyme is not a factor in the determination of the IC_{50} (page 127; Cornish-Bowden, 2004). Thus, the lack of a large increase in IC_{50} despite the very large increase in protein level is not unusual, in hindsight.

Creation of *OPB* null mutants was also successfully achieved in *L. major* promastigotes, as was shown by the lack of expression of OPB protein (Figure 5.12), showing that the peptidase is not essential for survival of this stage of the parasite *in vitro*. However, only two null mutant clones were produced out of a potential 33, a very low ratio of success. This could be because the promastigotes were initially disadvantaged by deletion of OPB and so only a low number of successful clones were able to survive the initial antibiotic selection. Alternatively, the OPB locus could be located in a section of the genome in which recombination was comparatively difficult, so the construct preferentially recombined into an alternative locus.

A lack of OPB did lead to a growth defect, with a lag in growth, slower growth rate and a lower stationary phase level of parasites (Figure 5.13), suggesting that the lack of OPB was in some way affecting the parasites. As would be expected with a non-essential gene, there was no change in the ploidy of the Δ*opb* clones, as

there was no need to alter the ploidy in order to preserve a WT copy of the gene (Figure 5.14).

OPB was confirmed to be responsible for almost all cleavage of Bz-R-AMC, as the Δ*opb* clones had only 0.7 to 1.4% of the activity of *L. major* WT promastigotes (Figure 5.15). There was a gene-dose response found, with the heterozygote having approximately half of the activity of the WT. This finding is in agreement with the dose response in enzymatic activity found when *OPB* was knocked out in *T. cruzi* (Caler et al., 1998). The ~1% of activity that was detected even in the Δ*opb* clones is most probably due to other enzymes with a very low level of ability to cleave this substrate or enzymes that are not present in the same compartment, the cytosol. The two other OPB-like peptidases present in *L. major*, OPB-like (LmjF06.0340) and Bem-46 (LmjF35.4020), could be responsible for the remaining activity. It is unknown what substrate specificity these peptidases have or where they are located within the parasite. Other potential enzymes involved are the cathepsin L-like peptidases CPB or CPC, which are located in the lysosome but should be able to cleave Bz-R-AMC, as the CPB isoform CPB2.8 has been found to preferentially cleave fluorogenic substrates after an arginine residue (Alves et al., 2001a). Only a very small amount of substrate may be delivered to this compartment through the endosomal system after being taken up by endocytosis.

As antipain had been previously found to be an anti-leishmanial compound (Coombs et al., 1982) and it was an inhibitor of the cleavage of Bz-R-AMC by OPB (Figure 3.3), it was hypothesised that the anti-leishmanial action could be due to the inhibition of OPB. A higher concentration of antipain was needed to kill the over-expresser lines, suggesting that more antipain was necessary to inhibit the extra OPB that was present (Figure 5.7). However, as it was possible to knock out OPB in *L. major*, it was unlikely that OPB would be the only target of antipain. It was indeed found that antipain could kill Δ*opb* clones, with the heterozygotes and Δ*opb* clone 21 having a lower LD_{50} than *L. major* WT, but Δ*opb* clone 10 having a very similar LD_{50} (Figure 5.16). This suggests that the other targets of antipain that are present in *Leishmania* may be expressed at a higher concentration in clone 10 to account for the increased LD_{50} of antipain when compared to the heterozygote population 1 from which it was derived.

The Δ*opb* clones were found to be similarly infective to PEM as *L. major* WT after 1 day of infection, suggesting that they are not defective in their ability to invade

macrophages. However, the percentages of infected cells declined over the 6 day infection period, with only around 5-10% infected by the end point of the experiment (Figure 5.17). This is a promising finding, suggesting there may be a defect in metacyclogenesis or differentiation to amastigotes associated with loss of OPB, or perhaps a reduced ability of amastigotes to survive. Metacyclic promastigotes are acknowledged as the infective stage of *Leishmania* (da Silva and Sacks, 1987) and so a decreased ability to survive in macrophages is indicative of a lower proportion of metacyclic promastigotes in the Δ*opb* clones. It was indeed found that there was a reduction in the number of metacyclic promastigotes in the Δ*opb* clones using two different methods: peanut lectin agglutination of non-metacyclic promastigotes and HASPB expression as assessed by western immunoblots (Table 5.3 and Figure 5.18). Δ*opb* clone 10 had a slightly higher number of macrophages still infected at day 6 post infection compared to Δ*opb* clone 21, and this was corroborated by the numbers of metacyclic promastigotes found using both methods, with Δ*opb* clone 10 having higher levels of metacyclics. However, since some metacylic promastigotes are produced by both null mutant clones, the very poor survival of amastigotes in macrophages suggests that OPB also has a role in either differentiation to amastigotes or in amastigote survival itself.

A few other genes known to be involved in metacyclogenesis have been described, including two cysteine peptidases. *L. major* promastigotes which lack sphingolipids cannot complete metacyclogenesis (Zhang et al., 2003; Denny et al., 2004). *L. mexicana* double null mutants for the cysteine peptidases CPA and CPB fail to transform to metacyclic cells, with inhibition of autophagosome degradation, and have a defect in infection of macrophages (Williams et al., 2006). A reduced ability to infect mice was also associated with lack of both sphingolipids and the cysteine peptidases, suggesting this is also associated with fewer metacyclics.

The Δ*opb* clones were also initially less infective to mice, with lesions developing in infected footpads with a lag period compared to wild-type parasites (Figure 5.19). Additionally, lesions developed at a slightly slower rate, with the lesions reaching a size of 5 mm between one and four weeks later than the mice infected with *L. major* WT. Lesions of Δ*opb* clone 10 were faster to develop than those of clone 21, echoing the infectivity to macrophages. This suggests that a lag in initiation of a lesion may be due to fewer metacyclic promastigotes being injected

or also to a reduced ability to differentiate to amastigotes, given that OPB has been shown to be upregulated in *L. braziliensis* and *L. donovani* amastigotes (Gamboa et al., 2007; Rosenzweig et al., 2008). To determine which is correct it would be necessary to repeat the macrophage and mice experiments infecting with isolated metacyclic promastigotes. Δ*opb* clones re-expressing OPB are also needed to confirm the results are due to lack of OPB.

5.3.1 Conclusions

OPB can be over-expressed in *L. major* and the over-expressing lines show a highly increased rate of cleavage of Bz-R-AMC. OPB can also be knocked out in promastigotes, showing it is not an essential gene in this life cycle stage. OPB is the enzyme responsible for the vast majority of Bz-R-AMC cleavage since a 99% reduction in cleavage activity was found in the Δ*opb* clones compared to the wild-type.

Δ*opb* clones showed reduced ability to survive in macrophages and a lower level of metacyclic promastigotes in stationary phase cultures. Lack of OPB is thus inhibiting the ability of the parasite to transform into the mammalian-infective stage of the parasite. Δ*opb* clones also had a lag in the growth of lesions in BALB/c mice, corroborating there being a deficiency in the ability to produce infective-stage parasites. That this result is due to the lack of OPB will have to be confirmed using Δ*opb* clones re-expressing OPB.

Chapter 6

USE OF LUCIFERASE AS A REPORTER SYSTEM FOR IDENTIFYING *LEISHMANIA*

6.1 Introduction

Any experimental study on *Leishmania* depends on accurate detection of the parasites in some form. In particular, any inaccuracy in determination of the number of *Leishmania*-infected macrophages presents a major hurdle to i) the development of anti-parasite drugs, ii) the determination of the effect of natural resistance or purposely inserted mutations on drug efficacy, and iii) the evaluation of the influence on macrophage infectivity of gene mutations and deletions (Croft et al., 2006). In order to test the effectiveness of potential anti-*Leishmania* drugs, a quick, quantitative and realistic assay is clearly desirable (Sereno et al., 2007).

6.1.1 Traditional methods for detection of *Leishmania*

In many drug screening studies, cultured promastigotes have been used for primary screens as these are amenable to a high throughput system with plate-based assays to determine viability, for example assays with MTT (Dutta et al., 2005; Tanaka et al., 2007) and alamar blue (Mikus and Steverding, 2000; Dutta et al., 2005). Such assays utilising promastigotes are advantageous due to their speed, accuracy and ease of use. However, the promastigote stage is not the human infective life-cycle stage (Neal and Croft, 1984) and so using promastigotes may result in potentially useful compounds being missed. For example, the pentavalent antimonial compounds used against *Leishmania*, sodium stibogluconate and meglumine antimoniate, are inactive against promastigotes (Neal and Croft, 1984).

Assays using axenically grown amastigotes have also been developed (Callahan et al., 1997; Sereno and Lemesre, 1997; Sereno et al., 2005). These have the advantage over promastigotes of being the human infective life-cycle stage, whilst still being quick, amenable to high throughput and easy to work with (Sereno et al., 2007). However, using axenic amastigotes obviously does not mimic the intracellular environment of *Leishmania* infection, with any role played by the host cell left unevaluated (Buckner and Wilson, 2005). Therefore, although axenic amastigotes have many advantages, it is still common practice to test anti-leishmanial compounds on infected macrophages (Yardley et al., 2006).

In most studies using macrophages, to determine the activity of anti-leishmanial compounds or of relative infectivity of genetically manipulated parasites, the

percentage of infected macrophages and number of amastigotes per macrophage are determined by manual counting; a labour intensive and somewhat subjective method (Neal and Croft, 1984; Sereno et al., 2007). Staining may also give inaccurate results as it can be very difficult to determine the viability of the amastigotes, and dead parasites may be included in the count (Sereno et al., 2007). Therefore, the development of alternative methods for the evaluation of the infection rates of *Leishmania* in macrophages has been an important area of study over recent years. Within this area, reporter gene systems have been extensively investigated (Naylor, 1999; Sereno et al., 2007).

6.1.2 Reporter gene systems

A reporter gene is defined as a gene with a measurable phenotype, allowing detection above a background of endogenous proteins (Naylor, 1999). Reporter gene systems are generally more sensitive than classical methods of screening *Leishmania* (Sereno et al., 2007). Reporter systems have been designed which use several different proteins, including β-galactosidase and β-lactamase (Buckner and Wilson, 2005), green fluorescent protein (Naylor, 1999), and luciferase, typically firefly (*Photinus pyralis*) luciferase (Gould and Subramani, 1988; Naylor, 1999).

6.1.2.1 β-galactosidase and β-lactamase

The bacterial enzyme β-galactosidase can be used in simple colorimetric and chemiluminescent assays (Naylor, 1999) and *E. coli* β-galactosidase has been stably expressed in *L. major* (LeBowitz et al., 1990). β-galactosidase has been reported to have high background activity in eukaryotic cells, including macrophages (Buckner and Wilson, 2005), so it may be unsuitable for evaluating *Leishmania*-infected macrophages. However, β-galactosidase has been expressed in *L. amazonensis*, by ectopic transfection, and detected by a change in absorbance upon cleavage of the β-galactosidase substrate CPRG (chlorophenol red β-D-galactopyranoside). Correlations were found both between numbers of promastigotes present and the absorbance recorded upon breakdown of CPRG and between the number of amastigotes in infected macrophages and the absorbance (Okuno et al., 2003).

β-lactamase has been suggested to be a better reporter gene than β-galactosidase as it is suitable for use in live cell assays and catalyses the degradation of various coloured substrates, meaning colorimetric assays are possible, with no problem of background enzymatic activity in eukaryotic cells (Moore et al., 1997; Campbell, 2004). *L. major* and *L. amazonensis* have been generated with β-lactamase integrated into their genomes and these cells were used to test anti-leishmanial compounds on infected murine macrophages, with a correlation found between both manual counts of the infections obtained and with published accounts of LD_{50} values for sodium stibogluconate and amphotericin B (Buckner and Wilson, 2005).

6.1.2.2 Green fluorescent protein

Green fluorescent protein (GFP), isolated from the *Aequorea victoria* jellyfish, is autofluorescent, which provides for easy measurement of its activity in living cells, and GFP has been used as a reporter in many species (Naylor, 1999). GFP has the advantage of its fluorescence range being highly sensitive and specific and also of being suitable for macrophage infection assays, as there is very little auto-fluorescence in the host cell (Zhao et al., 2006).

GFP has been extensively used for many purposes in *Leishmania*, including using GFP-expressing *L. donovani* promastigotes for anti-leishmanial drug testing (Singh and Dube, 2004). In this study clinical isolates were transfected with a cytoplasmic GFP expression vector and the effectiveness of anti-leishmanial drugs was evaluated by detecting the living cells using fluorescence-activated cell sorting (FACS) analysis. Also, GFP expressing *L. infantum* amastigotes have been used to test for the effect of HIV-1 infection on parasite ability to infect macrophages and grow intracellularly (Zhao et al., 2006), with the infectivity also evaluated using FACS analysis. The necessity of the use of FACS for detection of fluorescence from GFP-expressing *Leishmania* does, however, mean that there is a limited scope to use GFP as a reporter gene for fast, simple assays of the infectivity to macrophages as the fluorescence is not high enough for microplate reader detection (Sereno et al., 2007). Fluorescence signals from *L. amazonensis* expressing enhanced GFP (eGFP) have, however, been detected using a microplate reader, with a correlation found between numbers of promastigotes present and fluorescence and between amastigotes in infected macrophages and fluorescence (Okuno et al., 2003). This suggests that the stronger signal produced with eGFP is more applicable for monitoring *Leishmania* numbers.

6.1.2.3 Use of luciferase to detect pathogens

Firefly luciferase has been extensively used as a reporter system with many pathogens, including intracellular bacteria and other parasitic species. Since its luminescence depends on the presence of ATP in live cells, it is suitable as a test of cellular viability (Deb et al., 2000). Firefly luciferase is particularly suitable for intracellular detection because its substrate, luciferin, can rapidly diffuse into cells and tissues (Hutchens and Luker, 2007).

The survival of *Mycobacterium* species within macrophages upon administration of antimycobacterial agents, or stimulation of the macrophages, has been extensively studied with luciferase reporters. The intracellular survival of luciferase-expressing *M. tuberculosis* and *M. bovis* was investigated using THP-1 cells and antimycobacterial drugs (Arain et al., 1996). The results were validated by comparison to those obtained using traditional assessment methods (i.e., the assessment of colony forming units (CFU) of the bacteria released from macrophages, following drug treatment). The stimulatory effect of 19 cytokines applied to monocyte-derived macrophages pre-infection has also been successfully evaluated, using luciferase-expressing *M. bovis* (Bonay et al., 1999). The assay was validated by finding a linear relationship between the numbers of bacteria found by CFU measurement and the luminescence recorded. It was found to be both simple, and very sensitive to the viability of the mycobacteria. *M. aurum* expressing firefly luciferase have also been used to screen anti-mycobacterial drugs, at their reported minimum inhibitory concentrations, using infected J774A.1 macrophage cells, with a strong correlation between the luminescence and CFU measurement (Deb et al., 2000).

In the case of parasitic species, firefly luciferase has been used for *in vivo* imaging of *Plasmodium bergei* and *Toxoplasma gondii*. *P. bergei* expressing the luciferase gene were used to visualise the sequestration of infected erythrocytes in mice using both whole body and isolated organ imaging and with luciferin administered to generate luminescence (Franke-Fayard et al., 2005; Franke-Fayard et al., 2006). A novel site for sequestration was found, adipose tissue, with the sequestrations confirmed by comparison with histological slides, thus demonstrating the usefulness of luciferase in visualising host-parasite interactions (Franke-Fayard et al., 2005).

T. gondii was transfected with both firefly luciferase and click beetle luciferase, in order to investigate differences in the disease progression and dissemination of parasites in mice upon variation of the oral infection method (Boyle et al., 2007). Firefly luciferase-expressing *T. gondii* have also been used to demonstrate the difference in growth and dissemination of parasites with high or low virulence, with parasite presence determined from non-invasive images produced after injection of luciferin into live mice (Saeij et al., 2005). In this study the use of an anti-*Toxoplasma* compound, sulfadiazine, to control infection with virulent *T. gondii* was also investigated. The progress of infection was compared to untreated mice, demonstrating that drug effectiveness could be analysed using luciferase in an *in vivo* setting.

6.1.2.4 Use of luciferase to detect *Leishmania*

In *Leishmania*, firefly luciferase has been employed by using constructs that introduce the protein via both integration of the gene into the parasite genome at a defined locus and also having it as an ectopic copy (Sereno et al., 2007). The use of luciferase for parasite detection has so far been reported for six *Leishmania* species, both visceral and cutaneous, i.e., *L. donovani*, *L. infantum*, *L. major*, *L. panamensis*, *L. tropica* and *L. amazonensis*.

In 2000, both ectopic and integrated copies of firefly luciferase were introduced into *L. major* and *L. donovani* promastigotes and *L. infantum* axenic amastigotes, to allow the accurate quantification of amastigotes within macrophages (Roy et al., 2000). By using serially diluted parasites, a linear correlation was found between the number of *L. major* or *L. donovani* promastigotes present and the luminescence. Notably, promastigotes containing the ectopic luciferase had higher luminescence readings and required fewer parasites for detection (Roy et al., 2000). *L. infantum* axenic amastigotes expressing luciferase ectopically were also used to test the effect of antimonial drugs and pentamidine, and similar 50% inhibition concentration (IC_{50}) values were found using luciferase as have previously been reported using such tests as the MTT assay (Sereno et al., 2001).

L. donovani promastigotes have been generated that express a form of luciferase mutated at the C-terminal, so that it is retained in the cytosol rather than being transported into the glycosome – which results in a higher level of luciferase activity in the cells (Luque-Ortega et al., 2001). The luminescence activity was

again found to be consistent with the numbers of promastigotes present. These promastigotes were then used to evaluate the effect of naphthoquinones on intracellular ATP concentrations, as a measure of their effect on mitochondrial ATP production, with two of the three drugs found to have similar 50% effective dose (ED_{50}) concentrations when recorded via luminescence readings, mitochondrial membrane potential and parasite proliferation as assessed by MTT assay. Another 14 drugs were also tested with six found to inhibit luminescence, again with a good correlation with the parasite proliferation and mitochondrial membrane potential. Luminescence inhibition was found to correspond well with published data for anti-leishmanial activity for the effective drugs and the assay was postulated to be useful for investigating drugs with activity against ATP metabolism (Luque-Ortega et al., 2001).

6.1.2.4.1 Use of luciferase in infected macrophages

Over the last eight years, luciferase has been expressed in four species of *Leishmania* to evaluate the value of luminescence parasites for use in infected macrophage assays. In 2000, *L. infantum* axenic amastigotes, expressing luciferase from an ectopic construct, were used to infect THP-1 cells and a good correlation was found between the numbers of infected macrophages, and the parasites present, and the luminescence, as long as the loss of macrophages due to high parasitaemia was taken into account (Roy et al., 2000). If lower ratios of parasites to macrophages were used then the problem of loss of macrophages could be removed, but it was suggested by Roy et al. that to be confident that the numbers of parasites present were accurately determined it would be preferable to quantify the numbers of host cells using a second marker protein.

The same *L. infantum* axenic amastigotes have also been used to test the effect of antimonial drugs against infected THP-1 cells, with the luminescence levels detected compared to counting of the percentages of infected cells. The resulting IC_{50} values were similar to those previously reported in the literature (Sereno et al., 2001).

L. donovani promastigotes have been transfected with luciferase in several laboratories and used to test the usefulness of the cells for determining the infections in macrophages. For example, in 2000 *L. donovani* promastigotes, with the same ectopic construct expressing luciferase as in the above two studies with

L. infantum, were used to infect J774 macrophages and the numbers of parasites present were compared by manual counting and luminescence activity (Roy et al., 2000). With the *L. donovani* infected macrophages, a correlation between parasite number and luminescence was found, without any revision being necessary to account for macrophage loss.

An ectopic construct containing luciferase has also been transfected into *L. donovani* field isolates, both sodium stibogluconate-resistant and -sensitive, and a reference strain for comparison (Ashutosh et al., 2005). The luminescence of these parasites was compared to the numbers of parasites and found to correlate, with a minimum of 10 parasites needed for detection. The luminescence was maintained for up to 30 days without antibiotic pressure and a correlation was also found between the numbers of amastigotes counted in infected J774 macrophages and the luminescence. Infected macrophages were then used to compare the effectiveness of potassium antimony tartrate trihydrate (SbIII) against the resistant and sensitive strains, compared to the reference strain. The resistant strains were found to have threefold higher resistance compared to the sensitive strains and the reference strain, mirroring the clinical findings (Ashutosh et al., 2005).

In 2006, in another study using *L. donovani*, promastigotes expressing the ectopic construct of Roy et al. (Roy et al., 2000) were generated and used to investigate the effectiveness of topoisomerase inhibitors and standard anti-leishmanials in infected U-937 macrophage assays (Jean-Moreno et al., 2006). Luminescence was recorded and compared to counted parasite numbers, with a linear correlation found. ED_{50} values were then calculated for the compounds by luminescence activity, again with correlation to those found by counting of infected cells.

The ectopic construct of Roy et al. (Roy et al., 2000) has also been used to produce *L. panamensis* which express luciferase. These were used to infect U-937 macrophages and the ED_{50} of seven fluoroquinolones against intracellular amastigotes were determined by reading luciferase activity, to compare to the ED_{50} against macrophages as a measure of selectivity. The ED_{50} values determined by luminescence were found to be in good agreement with those determined by microscopical analysis of infected cells (Romero et al., 2005).

Finally, *L. tropica* promastigotes expressing luciferase have also been used in infected macrophages, using luminescence readings to compare the effectiveness of meglumine antimoniate against resistant and sensitive field isolates. THP-1 cells were infected with a number of sensitive and resistant parasites containing a luciferase ectopic construct and the ED_{50} values were determined for meglumine antimoniate. These were compared to those already determined by counting of parasite numbers and found to be very similar (Hadighi et al., 2006) and it was confirmed that clinically-resistant isolates had higher ED_{50} values than clinically-sensitive isolates.

6.1.2.4.2 Use of luciferase in infected mice

L. major promastigotes, containing ectopically-expressed luciferase, were used to investigate footpad infection of BALB/c mice. The luminescence of pulverised footpad tissue was compared to an estimation of the numbers of parasites by a limiting dilution assay. A correlation was found between parasite number and luminescence for two weeks post-infection. However, by four weeks, the luminescence was underestimating the number of parasites due to the loss of the ectopic construct containing the luciferase when antibiotic pressure could not be maintained *in vivo*, since copy numbers of the ectopic construct were found to decrease over time. *L. major* promastigotes, with luciferase integrated into the ribosomal locus, have also been used for footpad infection of BALB/c mice to compare with the ectopically expressing *L. major*. With these parasites, a correlation was found between parasite number and luminescence for eight weeks post-infection (Roy et al., 2000).

L. donovani promastigotes, containing ectopic luciferase, have also been used in animal experiments, using BALB/c mice, with the luminescence of pulverised liver compared to the number of parasites, estimated from counting parasites on impression smears, over an eight week period. A slight underestimation of numbers was again found by luminescence, with the underestimation increasing from week 2 to week 6 of infection, which again was ascribed to the loss of the ectopic construct containing the luciferase when there was no antibiotic pressure (Roy et al., 2000).

Luciferase has also been used for *in vivo* imaging of infected mice. In this study, 1×10^6 *L. amazonensis* promastigotes, stably expressing luciferase integrated into

the 18S rRNA locus of the genome, were inoculated into the ear of BALB/c mice and over a two week period the luminescence recorded was compared to limiting dilution assays. The luminescence was induced by the injection of luciferin into the mice. Parasites could be detected by one day post-infection. The number of parasites found by limiting dilution assays increased over the period, particularly quickly after 1 week. This was replicated in the luminescence readings, with a large increase in luminescence at day 16 compared to day 8. It was found that inoculating with 1 x 10^5 parasites was also detectable, but when only 1 x 10^4 parasites were used no luminescence was found (Lang et al., 2005). The luminescence readings correlated with the numbers of parasites found by limiting dilution. It was also found that it was possible to use standard curves for luminescence of purified amastigotes in order to quantify the number of parasites present in either infected macrophages or mouse tissues (Lang et al., 2005).

6.1.2.5 General consensus on the effectiveness of luciferase for detection of *Leishmania*

All the published studies which have been reviewed suggest that the use of firefly luciferase as a reporter for the presence of *Leishmania* in infected macrophages is a viable prospect and that the speed and accuracy of evaluation of both anti-leishmanial compounds and infectivity of genetically altered parasites could be improved by the use of luminescent *Leishmania*. It should also potentially be useful for more accurate determination of whether amastigotes are alive within macrophages, which is not always possible using traditional staining methods.

For this reason a short digression was made from the main research here reported to examine the potential value of luciferase-expressing *Leishmania* for monitoring differences in parasite infectivity to macrophages in the main work.

6.2 Results

6.2.1 Luciferase lines used

Two constructs were used to generate luciferase-expressing *Leishmania*; one was for integration into the *Leishmania* genome and the other was for ectopic expression.

6.2.1.1 Construct for integration of luciferase reporter

The integrating construct was a gift of Prof. D.F. Smith (University of York, UK). It was designed to integrate into the ribosomal RNA small subunit of *Leishmania*. The construct was derived from the pSSU-int construct of Dr T. Aebischer of the University of Edinburgh, UK, as described in Misslitz et al., 2000.

The vector, pGL1313 (Figure 6.1), was prepared for transfection by enzymatic digestion using the restriction enzymes *Ase* I and *Pme* I by Dr Lesley Morrison, University of Glasgow. *L. major, L. mexicana* and *L. infantum* parasites were species-checked by the PCR method (also by Dr Lesley Morrison, University of Glasgow), and then transfected with the digested pGL1313 DNA, as described in Section 2.5.7.

Transfectants were selected with hygromycin, as described in Section 2.1.1, and the parasites were then diluted down and plated in three 96 well plates, to give an average of 0.1 parasites per well in order to generate clones. Two clones were selected for each species and tested for luminescence.

6.2.1.2 Construct for ectopic expression of luciferase reporter

The ectopic construct was a gift of Prof. M. Ouellette (McGill University, Montreal, Canada). It had been used previously in *L. major*, *L. donovani* and *L. infantum* (Roy et al., 2000; Sereno et al., 2001; Jean-Moreno et al., 2006), with the neomycin resistance gene, and in *L. tropica* with the hygromycin resistance gene (Hadighi et al., 2006). The vector map is shown in Figure 6.2. The intergenic region of the alpha tubulin gene is included to control the maturation of the luciferase transcript (Roy et al., 2000) and the 3' UTR of *L. infantum* amastin is included in the construct to induce reporter-gene expression specifically in amastigotes (Boucher et al., 2002). The ectopic construct was transfected into *L. mexicana, L. major* and *L. infantum* cells. Selection of clonal cell lines was not necessary since the luciferase reporter was ectopic; as such the cells were kept as populations.

The *Leishmania* parasites containing the luciferase ectopic construct will hereafter be referred to as LUC cells.

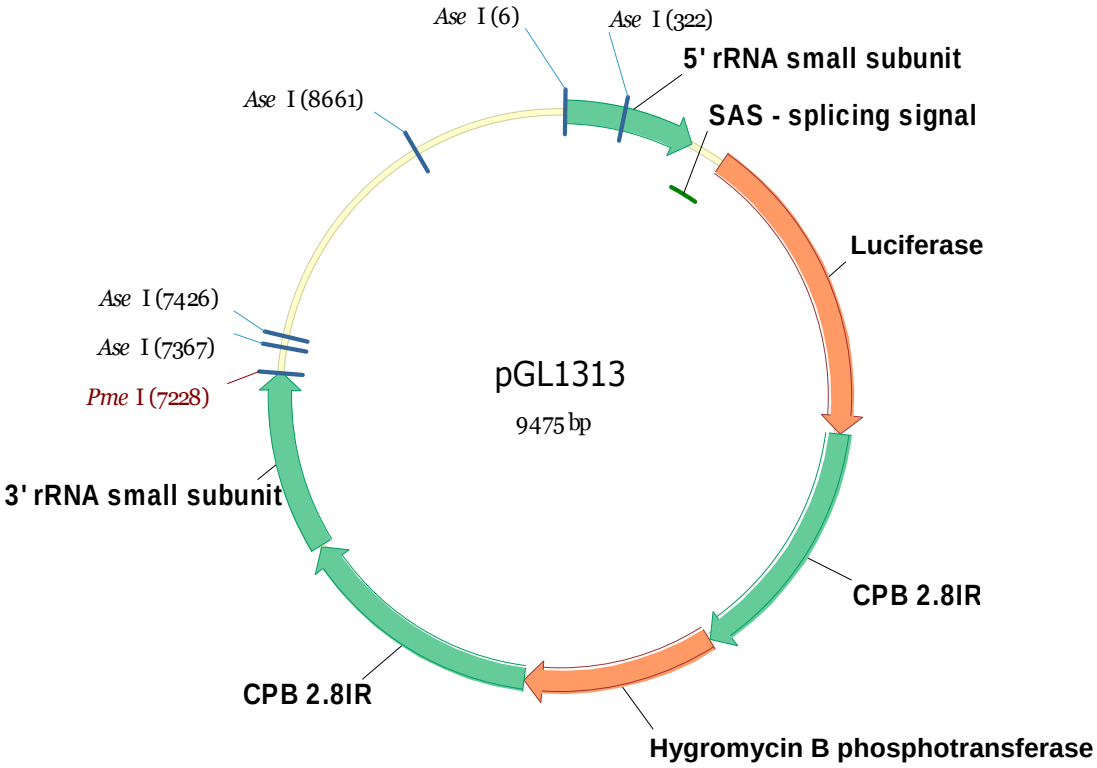


Figure 6.1 Integrating construct for the expression of luciferase in *Leishmania*. Map of the integrating construct, pGL1313. The 5' and 3' rRNA small subunit sections are included for integration into this locus, the CPB 2.8 intergenic regions (IR) are to ensure high level of expression in the amastigote stage and the SAS is the splice acceptor site and *Ase* I and *Pme* I indicate the sites where digestion occurs with these restriction enzymes.

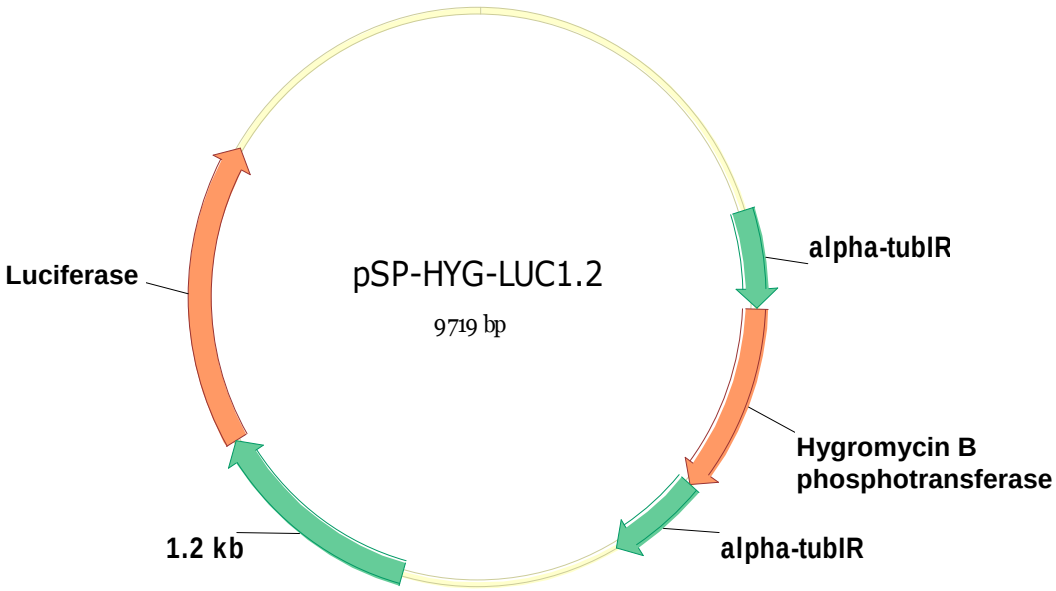


Figure 6.2 Ectopic construct for the expression of luciferase in *Leishmania*. Map of the ectopic construct, pSP-HYG-LUC1.2. Luciferase indicates the luciferase gene, 1.2kb is the last 1.2 kb of the amastin 3' untranslated region and alpha-tubIR is the intergenic region of the α-tubulin gene.

6.2.2 Detection of *Leishmania* using integrating construct

6.2.2.1 Validation of luciferase expression

After the generation of hygromycin resistant clonal lines they were investigated to determine whether, or not, they expressed the luciferase gene and were thus luminescent.

A final concentration of 12.5 µM D-luciferin was administered to the two clonal lines of each species and it was found that luciferase was being expressed by five of the *Leishmania* promastigote clonal lines: both of the *L. mexicana* and *L. infantum* clones, and clone 2 of *L. major*, since increasing luminescence was recorded with increasing numbers of these cells (Figure 6.3).

6.2.2.2 Validation of detection of promastigotes

Two methods were compared for the detection of promastigotes, i) the addition of D-luciferin to cells to a final concentration of 12.5 µM, and ii) Roche's Luciferase Reporter Gene Assay, constant light signal, kit (hereafter referred to as the luciferase assay kit).

There was a linear correlation between increasing numbers of cells and the increase in luminescence (Figures 6.4 and 6.5). The clonal lines were in the same order of luminescence with both detection methods: the two *L. infantum* clones and *L. mexicana* clone 2 with the highest luminescence, followed by *L. mexicana* clone 1 and lastly *L. major* clone 2.

The luciferase assay kit was found to be more sensitive, generating luminescence readings around 25-40 times higher than those obtained with D-luciferin.

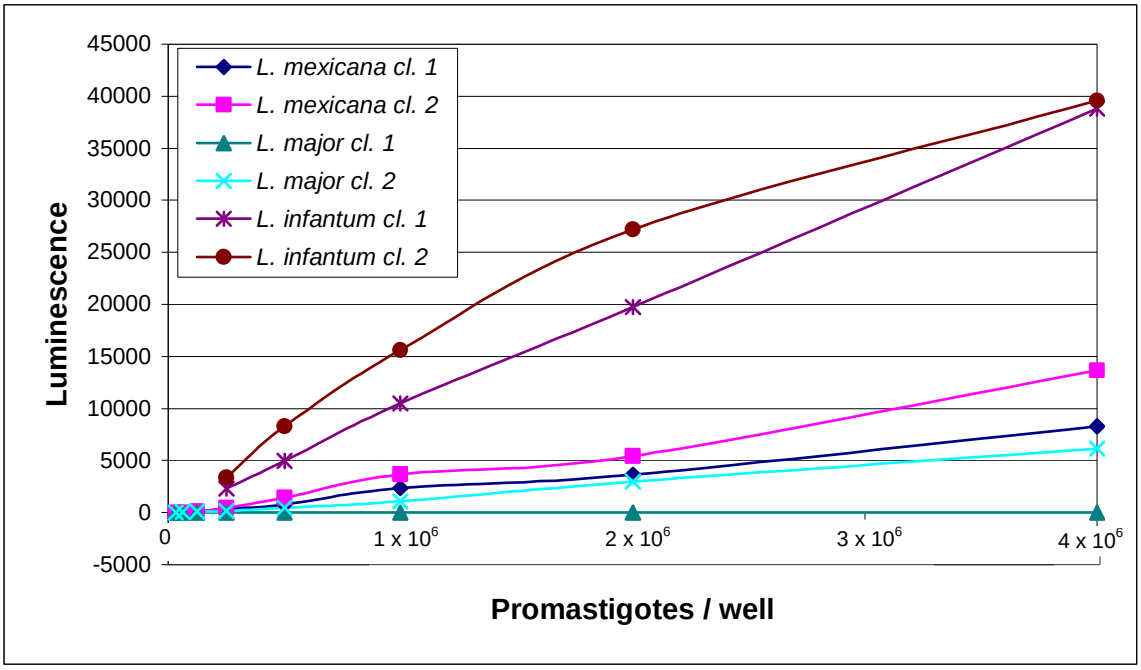


Figure 6.3 Use of D-luciferin to detect the luminescence of *Leishmania* promastigotes. 20 µl of a 1 mM stock solution of D-luciferin was administered to 180 µl of log phase promastigotes, giving a final concentration of 12.5 µM D-luciferin. Luminescence was recorded for two replicates per concentration of cells and a mean of these is presented. cl. = clone.

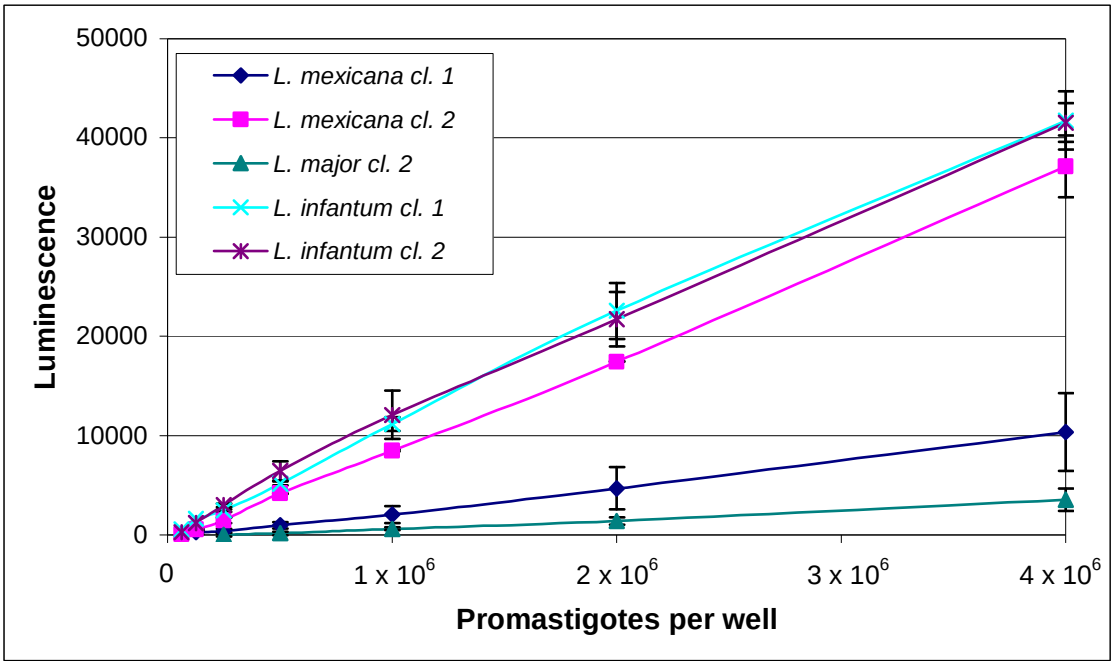


Figure 6.4 Use of D-luciferin to detect luminescence of *Leishmania* luciferase clonal log phase promastigotes. 20 µl of a 1 mM stock solution of D-luciferin was administered to 180 µl of log phase promastigotes, giving a final concentration of 12.5 µM D-luciferin. Luminescence was recorded for three experiments (each of a single replicate), and a mean of these is presented. cl. = clone.

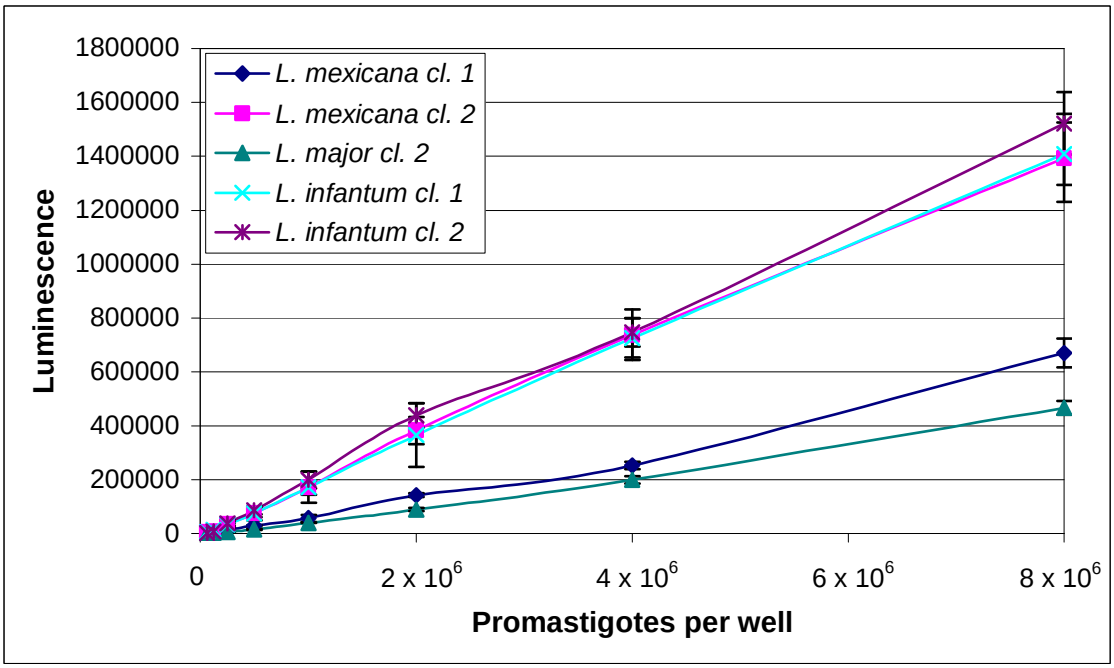


Figure 6.5 Use of the luciferase assay kit to detect the luminescence of *Leishmania* luciferase clonal log phase promastigotes. 100 µl of the luciferase assay kit solution was added to 100 µl of cells. Luminescence was recorded for three experiments (each of a single replicate), and a mean of these is presented. cl. = clone.

6.2.2.3 Validation of detection of amastigotes in infected peritoneal exudate macrophages (PEM)

Luminescence was compared with the percentage of infected cells and the average number of amastigotes per macrophage, both as determined by manual counting (Tables 6.1 – 6.3). The luciferase assay kit was used to analyse luminescence in infected macrophages since adding D-luciferin did not generate luminescence above background levels.

Luminescence readings were highly variable and did not correspond to either the percentages of cells that were infected or to the numbers of parasites found per macrophage. For example, for the *L. mexicana* clone 1 there was a 14-fold variation in the luminescence readings in the 4th and 5th experiments, when the macrophages were similarly infected, viz., 72% and 68% infected with 2.8 and 3.8 amastigotes per macrophage respectively.

Table 6.1 Corrected mean luminescence readings found for PEM infected with *Leishmania* luciferase clonal lines, using the luciferase assay kit.

Exp^t	*L. mexicana* clone 1 (± SEM)	*L. mexicana* clone 2 (± SEM)	*L. major* clone 2 (± SEM)	*L. infantum* clone 1 (± SEM)	*L. infantum* clone 2 (± SEM)
1	6,848 ± 1,260	286 ± 137	325 ± 40	76 ± 23	-12 ± 2
2	464 ± 68	1,747 ± 420	52 ± 8	13 ± 4	743 ± 59
3	70 ± 6	267 ± 38	67 ± 13	8 ± 6	15 ± 7
4	29,884 ± 2,208	51,710 ± 6,934	379 ± 58	357 ± 15	109 ± 19
5	2,095 ± 243	2,014 ± 401	242 ± 33	2,046 ± 604	5,796 ± 1,956

Table 6.2 Mean percentages of infected macrophages found for PEM infected with *Leishmania* luciferase clonal lines.

Exp^t	*L. mexicana* clone 1 (± SEM)	*L. mexicana* clone 2 (± SEM)	*L. major* clone 2 (± SEM)	*L. infantum* clone 1 (± SEM)	*L. infantum* clone 2 (± SEM)
1	49 ± 10	38 ± 7	74 ± 5	69 ± 4	78 ± 2
2	33 ± 2	42 ± 7	48 ± 8	80 ± 3	41 ± 6
3	47 ± 4	54 ± 6	56 ± 8	94 ± 2	95 ± 2
4	72 ± 6	87 ± 3	90 ± 2	78 ± 7	77 ± 7
5	68 ± 5	76 ± 11	78 ± 3	66 ± 4	66 ± 8

Table 6.3 Mean number of amastigotes found per macrophage for PEM infected with *Leishmania* luciferase clonal lines.

Exp[t]	*L. mexicana* clone 1 (± SEM)	*L. mexicana* clone 2 (± SEM)	*L. major* clone 2 (± SEM)	*L. infantum* clone 1 (± SEM)	*L. infantum* clone 2 (± SEM)
1	2.0 ± 0.5	0.8 ± 0.1	3.0 ± 0.6	2.3 ± 0.1	3.0 ± 0.1
2	1.1 ± 0.2	1.5 ± 0.3	1.6 ± 0.5	3.8 ± 0.6	1.2 ± 0.2
3	1.2 ± 0.2	1.8 ± 0.2	1.4 ± 0.4	9.1 ± 1.1	8.9 ± 1.0
4	2.8 ± 0.4	4.4 ± 0.4	3.9 ± 0.2	3.2 ± 0.7	2.3 ± 0.2
5	3.8 ± 0.3	5.0 ± 0.6	4.4 ± 0.9	2.6 ± 0.2	2.6 ± 0.9

6.2.3 Detection of *Leishmania* using ectopic construct

6.2.3.1 Validation of luciferase expression

LUC promastigote populations were investigated for expression of the luciferase gene and hence luminescence. For all experiments using the LUC cells the luciferase assay kit was used.

Luciferase was found to be expressed by each of the LUC populations (Table 6.4). The populations were thus used to determine suitability for detection of amastigotes within PEM.

Table 6.4 Corrected mean luminescence readings found with 3 x 10^6 cells for *Leishmania* LUC populations, using the luciferase assay kit.

Parasites tested	Luminescence of log phase cells (± SEM)	Luminescence of stationary phase cells (± SEM)
L. mexicana LUC	345,783 ± 30,890	247,018 ± 5,916
L. major LUC	386,730 ± 19,289	556,457 ± 76,838
L. infantum LUC	425,292 ± 23,014	248,701 ± 1,713

6.2.3.2 Longevity of luminescence without antibiotic selection

To determine the longevity of the retention of the luciferase ectopic construct and thus the luciferase gene, the luminescence was compared at several timepoints over a 24 day period using cultures with the antibiotic pressure removed and control cultures with the antibiotic pressure maintained.

Luciferase activity was detectable for seven days post antibiotic removal, in all lines, though the *L. mexicana* LUC cells lost luciferase activity and presumably the ectopic construct very quickly after this (Figure 6.6). Maintenance of luciferase activity for seven days should be sufficient for the experiments planned using infection of macrophages.

6.2.3.3 Validation of detection of amastigotes in infected PEM

PEM were infected with LUC stationary phase promastigotes and the luciferase assay kit was used to compare luminescence with the percentage of infected cells and the average number of amastigotes per macrophage, both determined by manual counting (Table 6.5).

Table 6.5 Comparison of the corrected mean luminescence readings with the percentage of macrophages infected and the number of amastigotes per macrophage.

Parasite and ratio of infection	Luminescence (± SEM)	% PEM Infected (± SEM)	No. amastigotes per PEM (± SEM)
L. mexicana 2:1	72.1 ± 11	80 ± 1.7	3.4 ± 0.2
L. mexicana 5:1	70.0 ± 4	83 ± 7.8	4.7 ± 0.5
L. major 5:1	4.5 ± 5	81 ± 2.7	5.5 ± 0.6
L. major 7:1	18.0 ± 9	86 ± 3.1	5.7 ± 0.4
L. infantum 7:1	26.0 ± 3	48 ± 3.8	0.9 ± 0.1
L. infantum 10:1	25.0 ± 6	78 ± 3.1	3.7 ± 0.7

Despite the macrophages being highly infected, no significant luminescence was recorded above background levels and no correlation was found between luminescence and the percentage of infected PEM and the number of amastigotes per macrophage. For example, for the *L. infantum* LUC population, there was a 30% difference in the percentage of macrophages infected and a 4 fold difference in the number of amastigotes present in the macrophages and yet no difference in luminescence was detected.

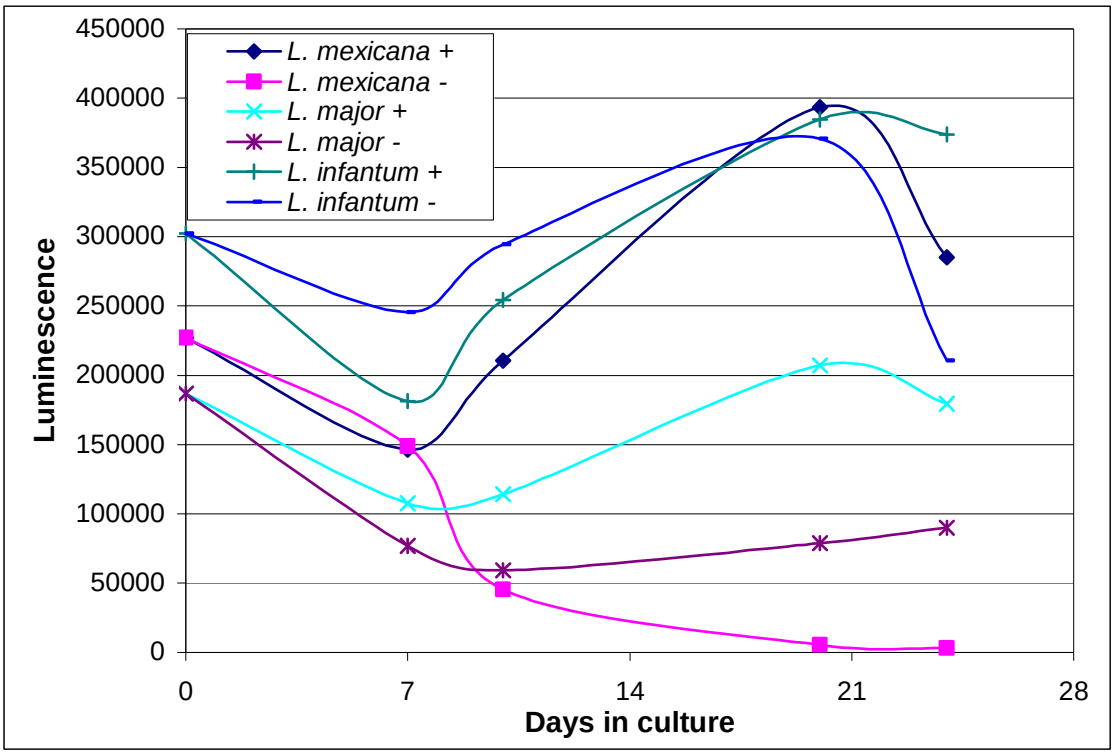


Figure 6.6 Corrected mean luminescence readings found for *Leishmania* LUC populations with or without hygromycin selective pressure. Luminescence was measured over 24 days in culture using the luciferase assay kit. + = presence of hygromycin, - = absence of hygromycin. 50 µl of luciferase assay kit solution was added to 150 µl of cells, with 3 x 10^6 cells used per replicate. Luminescence was recorded for three replicates per population and a mean of these is presented.

6.2.3.4 Determination of presence of luciferase

Given the lack of luminescence detected for infected macrophages, the potential loss of the luciferase ectopic construct as the parasites transformed from promastigotes to amastigotes within macrophages was investigated.

A comparison was made of luminescence readings as *L. mexicana* LUC promastigotes transformed to axenic amastigotes and back to promastigotes (Table 6.6). Promastigotes transforming to axenic amastigotes (AA) are sub-passage 0 (sp0), and were sampled over the week following inoculation into amastigote medium. Fully transformed amastigotes were found by the 7th day post inoculation. The amastigotes were sub-passaged again and sampled after a further week (sp1) before the cells were inoculated into promastigote medium and sampled after one week when they had returned to promastigote form.

Cell samples were also taken during the transformation for western immunoblot analysis with anti-firefly luciferase antibody, to determine levels of luciferase protein (Figure 6.7).

Table 6.6 Comparison of corrected mean luminescence readings recorded for *L. mexicana* LUC cells.

Cells used	Mean luminescence (± SEM)
Promastigotes	234,231 ± 3,361
Axenic amastigotes sp0 2nd day transforming	76,377 ± 240
Axenic amastigotes sp0 4th day transforming	16,512 ± 53
Axenic amastigotes sp0 7th day transforming	8,568 ± 138
Axenic amastigotes sp1	4,013 ± 107
Promastigotes transformed from axenic amastigotes	327,203 ± 4,846

There was a reduction in the luminescence recorded over the period of transformation from promastigotes to axenic amastigotes. However, the ectopic construct was not lost by the cells, as the luminescence was restored when transformed back from amastigotes to promastigotes.

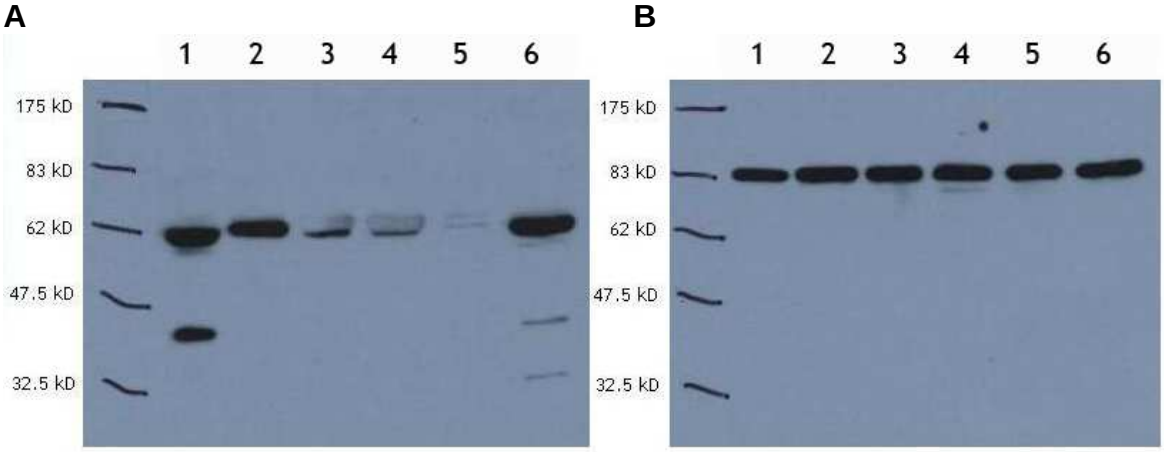


Figure 6.7 Western immunoblot of luciferase presence during *Leishmania* differentiation. A = luciferase presence, using anti-firefly luciferase mouse monoclonal antibody at 1 in 1000 dilution. B = oligopeptidase B (OPB) presence as a loading control, using purified anti-OPB sheep antibody at 1 in 20,000 dilution. HRP-conjugated anti-mouse and anti-sheep secondary antibodies were used at a concentration of 1 in 5,000. The chemiluminescence was detected in an Xomat processor. Promastigotes were transformed to axenic amastigotes (AAs) over one week, sub-passaged as amastigotes and transformed back to promastigotes. 1= *L. mexicana* promastigotes, 2= *L. mex* AA 2nd day transforming, 3= *L. mex* AA 4th day transforming, 4= *L. mex* AA 7th day transforming, 5= *L. mex* AA subpassage 1, 6= *L. mex* promastigotes transformed back from AA.

The level of luciferase protein decreased as the transformation process proceeded, although luciferase was never fully ablated as enzyme was detected in the sp1 axenic amastigotes. The enzyme was also seen to be highly expressed once more after the parasites transformed back to promastigotes, consistent with the luminescence readings obtained.

6.3 Discussion

Firefly luciferase has been shown to be a valuable tool for the detection of *Leishmania*, whether as promastigotes and intracellular amastigotes or *in vivo* (Roy et al., 2000; Jean-Moreno et al., 2006; Sereno et al., 2007), with six *Leishmania* species having been generated that express luciferase, by other laboratories.

Both the integrative luciferase construct and the ectopically expressed luciferase construct were successfully used to transfect promastigotes to produce luminescent *Leishmania* parasites of *L. mexicana*, *L. major* and *L. infantum* species (Figure 6.3 and Table 6.4). Five clones were produced using the integrative luciferase construct, two each for *L. mexicana* and *L. infantum* and one for *L. major*, and populations were produced of the ectopic luciferase, one per species (LUC cells).

Intuitively, it was thought that an integrative luciferase construct would be the most useful; as clones could be produced and the levels of luciferase should be very similar between parasites, whereas with ectopically expressed protein the luciferase expressed could be very different between cells based on the copy number of the plasmid in individual cells. However, the sensitivity of an integrative construct has previously been compared to an ectopic construct and the ectopic construct was found to exhibit higher luminescence and to be better suited for macrophage infections (Roy et al., 2000). Interestingly, in that work it was found that the amount of luciferase RNA expressed was similar for the parasites with the integrated gene and the ectopic gene, meaning some unknown factor was involved in determining luminescence levels.

Initially, a comparison was made between the use of D-luciferin and that of a lysis method, utilising Roche’s Luciferase Reporter Gene Assay, constant light signal, kit (luciferase assay kit), for detection of the integrated luciferase in promastigotes

(Figures 6.4 and 6.5). D-luciferin has previously been used to detect parasites in real time *in vivo* monitoring (Lang et al., 2005), and a modified luciferase ester to detect promastigotes (Luque-Ortega et al., 2001), whereas the luminescent cells have been assayed by lysis and an assay buffer in most studies, both of free living promastigotes (Roy et al., 2000; Sereno et al., 2001; Romero et al., 2005) and of infected macrophages (Ashutosh et al., 2005; Romero et al., 2005; Jean-Moreno et al., 2006).

It was found that the addition of D-luciferin was acceptable for the detection of luciferase expressing promastigotes and that a linear correlation existed between promastigote numbers and luminescence recorded (Figure 6.4). However, by using the luciferase assay kit much higher luminescence was recorded, with the same correlation of cell number and luminescence and the same order of luminescence activity in the 5 clonal lines (Figure 6.5).

With macrophage infections the application of D-luciferin to generate luminescence was not successful, since the luminescence found was barely above background levels. This was probably due to the luciferin having to cross the macrophage membrane into the parasitophorus vacuole and then into the amastigote before it could act as a substrate for the luciferase. Therefore only data from the use of the luciferase assay kit was presented (Table 6.1).

Percentages of infected macrophages were found to vary between 33% and 95%. There was very high variation found in the luminescence readings, even when there were similar numbers of amastigotes in similarly infected cells. For example, for *L. mexicana* clone 2 the luminescence readings ranged from 267 to 51,710 and the similarly infected last two experiments (87% and 76% infected, with 4.4 and 5.0 amastigotes per macrophage, respectively) had luminescence readings of 51,710 and 2,014. For *L. infantum* clone 1, two experiments had 80% and 78% infected macrophages, with 3.8 and 3.2 amastigotes per macrophage, but luminescence readings of 13 and 347 respectively.

Overall, for intracellular parasites generally low luminescence readings were recorded for the *L. major* clone and the *L. infantum* clones, relative to the *L. mexicana* clones. Although the *L. major* clone did have the lowest luminescence of all the clones when assessed as promastigotes, the low readings

are particularly unexpected for the *L. infantum* clones as these had the highest luminescence as promastigotes.

The *L. infantum* clones were also sent to the laboratory of Prof. D.F. Smith, who had supplied the original construct, where they were used to investigate the progress of mouse infections, but luminescence could not be detected above background in this case either. *In vivo* imaging was successfully completed using *L. donovani* parasites expressing the same luciferase construct, however, so it is possible that there may be a difference caused by the species used, perhaps because the CPB 2.8 intergenic region used to ensure expression in the amastigote stage has variable effectiveness for this purpose in different species.

Since there was a failure to correlate the percentage of cells infected and the luminescence readings (Tables 6.1 to 6.3), it was decided that there was no advantage in continuing with tests with these luminescent parasites as a method to accurately determine the number of infected macrophages. The ectopic construct was thus obtained from Prof. Ouellette, as it had been used by several other groups and success reported in the literature (Roy et al., 2000; Sereno et al., 2001; Romero et al., 2005; Hadighi et al., 2006; Jean-Moreno et al., 2006). Both cutaneous (*L. major*, *L. tropica* and *L. panamensis*) and visceral (*L. infantum* and *L. donovani*) luciferase-expressing lines had been successfully generated, though the *L. major* parasites had not been reported to have been used in macrophage infections.

Only one paper shows the calibration data used to correlate the numbers of amastigotes present and the luminescence (Roy et al., 2000). Other papers published using the ectopic construct merely show that ED_{50} values could be calculated that were very similar to those calculated by manual counting and those already published (Sereno et al., 2001; Romero et al., 2005; Hadighi et al., 2006; Jean-Moreno et al., 2006), and state that correlation was found.

The LUC populations were shown to express good levels of luminescence (Table 6.4) and to maintain the luminescence for at least seven days in the absence of hygromycin selective pressure (Figure 6.6). The *L. mexicana* and *L. major* promastigotes lost their luminescence from day 10 after the antibiotic was removed, whereas the *L. infantum* promastigotes maintained their luminescence until day 20. The week of continued luminescence in the absence of antibiotics

found with all three lines should be sufficient to ensure that the cells could be used for macrophage infections when antibiotic pressure is impossible to maintain.

Macrophages infected with the LUC populations were found to exhibit no luminescence above background, despite the high levels of infection of the macrophages (Table 6.5) and the previous finding of luminescence in promastigotes (Table 6.4). The lack of luminescence in amastigotes was unexpected, as the amastin 3' UTR of *L. infantum* was included in the construct to ensure the luciferase was expressed in amastigotes, giving increased mRNA accumulation in amastigotes. The last 770 bp of the amastin 3' UTR has been shown to increase luciferase activity by 13-fold in axenic amastigotes and 24.5-fold in intracellular amastigotes (Boucher et al., 2002).

It has been described previously that higher levels of luminescence were found with promastigotes in culture compared to intracellular amastigotes (Roy et al., 2000), and that a 10-fold increase in luminescence was found when axenic amastigotes were transformed into promastigotes. It has also been shown that *L. amazonensis* amastigotes extracted from bone-marrow derived macrophages gave 10-fold higher luminescence than amastigotes from mouse lesions, but that promastigotes differentiated from amastigotes released from the infected bone-marrow macrophages gave a 30-fold higher luminescence than the amastigotes (Lang et al., 2005). These previous reports would correlate with the lowered luminescence found with intracellular amastigotes, compared to promastigotes, in this work.

It has been postulated that the decrease in luminescence found in amastigotes could be due to degradation (Lang et al., 2005), as firefly luciferase has been suggested to be susceptible to proteolysis (Boucher et al., 2002). Alternatively, the ectopic construct could be lost by the parasites as they transformed to amastigotes. Thus the transformation process was examined with *L. mexicana* LUC axenic amastigotes, as an easier model to work with compared to macrophage-derived amastigotes. The data show that the ectopic construct was not lost, as there was luminescence in the promastigotes that were transformed back from amastigotes (Table 6.6). However the levels of luciferase protein were also shown to decrease over the transformation from promastigotes to amastigotes and to return to the original level once back in the promastigote stage (Figure 6.7), supporting the luminescence data obtained.

6.3.1 Conclusions

The integrating luciferase construct gave highly variable results with infected macrophages, with no consistency being found nor correlation between the numbers of amastigotes present and the luminescence.

Given that five studies had successfully used the luciferase-encoding ectopic construct in macrophage tests, it was expected that more success would be obtained by using this line. However, effectively no luminescence was detectable when macrophages were infected with the *L. major*, *L. mexicana* and *L. infantum* LUC cells, though good luminescence was obtained with promastigote LUC parasites. The ectopic construct was retained by *L. mexicana* axenic amastigotes; even though the luminescence recorded decreased and the luciferase protein was found to be present at a much lower level in the amastigotes. It can be supposed that the same is true of the amastigotes within infected macrophages.

Since most of the studies published to date have used a neomycin-resistant form of the construct, it is possible that the construct has been altered in some way when the resistance markers were swapped. This seems unlikely, however, since the hygromycin resistant plasmid has been successfully used to show differences in drug sensitivities of drug-resistant and -sensitive field isolates of *L. tropica* (Hadighi et al., 2006). Another possibility is that there is a species difference that can account for the inability to successfully use LUC parasites for detection of amastigotes within infected macrophages, as *L. major* and *L. mexicana* parasites have not been previously used, and the *L. infantum* parasites reported in the literature were transfected as axenic amastigotes, rather than as promastigotes, which could cause the difference in success (Sereno et al., 2001). This may also be true for the integrative construct given the reported inability to use *L. infantum* with integrated luciferase for *in vivo* imaging when *L. donovani* cells were successful.

Unfortunately, it was not possible to investigate the potential species issue in this study. However, it is suggested that the use of *L. donovani* parasites or transfecting axenic amastigotes might be fruitful lines for further research.

Chapter 7

GENERAL DISCUSSION

7.1 Analysis of Peptidase Activity in Live *Leishmania*

Prior to this study, the detection of peptidase activity in *Leishmania* using fluorogenic compounds had only been reported using cell lysates, although fluorogenic compounds had been used to investigate the peptidases in live cells of numerous mammalian cell lines (Boonacker and Van Noorden, 2001). I have now shown that peptide-based compounds containing the fluorochrome 7-amino-4-methylcoumarin (AMC) may successfully be used to detect the activity of peptidases in live *Leishmania*. Bz-R-AMC was the only peptide tested that was successfully hydrolysed by *Leishmania* promastigotes (Section 3.2.1). Bz-R-AMC was thus selected for this study since it is a small molecule with the potential to easily enter cells, with only one peptide bond able to be cleaved and the preliminary studies showed that it was readily hydrolysed by both *L. mexicana* and *L. major*.

Cleavage of Bz-R-AMC by live *Leishmania* promastigotes was shown to be due to a peptidase which, based on analysis of the inhibition profile, is a serine peptidase with trypsin-like specificity for cleavage after basic residues (http://merops.sanger.ac.uk). The absence of significantly different cleavage by the mutant line with lysosomal trafficking defects, the *L. major* VPS4^{E235Q} mutant (Besteiro et al., 2006), compared to equivalent wild-type promastigotes, suggested that the peptidase involved was unlikely to be resident in the lysosomal system, since in the VPS4^{E235Q} mutants the substrate would be unlikely to be properly delivered to the lysosomal compartment (Section 3.2.1). Preventing endocytosis, using the actin polymerisation inhibitor cytochalasin D, had no effect on the hydrolysis of Bz-R-AMC. Endocytosis is thus not the method by which Bz-R-AMC was entering *Leishmania*. This would also seem to suggest that the Bz-R-AMC was unlikely to be being hydrolysed in the endosomal/lysosomal system, and if it was, it was not entering it directly. It was also impossible to prevent the cleavage of Bz-R-AMC by blocking the arginine transporter; a transporter that perhaps could take up the substrate (Shaked-Mishan et al., 2006). These results together imply that the substrate is most probably entering the cell via diffusion and is being hydrolysed by a peptidase in the cytosol (Section 3.2.3).

As the enzyme being investigated had a trypsin-like specificity, given the likely location of the peptidase and the pattern of inhibition, it was hypothesised that the

peptidase likely to be responsible for the hydrolysis was oligopeptidase B (OPB, LmjF.09.0770). This hypothesis was shown to be correct when immuno-precipitation of OPB protein from *Leishmania* lysate resulted in prevention of cleavage of the peptide (Section 4.2.5).

OPB is present in trypanosomatids, plants and bacteria (Coetzer et al., 2008). It was first shown to be present in *L. major* in 1999 (Morty et al., 1999b), and subsequently in *L. amazonensis* (de Matos Guedes et al., 2007). In this study, OPB was found to be expressed at approximately equal levels in all three main life cycle stages in *L. major* (Section 4.2.1), whilst OPB has previously been found to be upregulated in amastigotes of *L. braziliensis* and *L. donovani* compared to promastigotes (Gamboa et al., 2007; Rosenzweig et al., 2008). There may be a difference in expression levels between species, although the methods used may account for the different findings. The higher expression reported for *L. braziliensis* amastigotes compared with promastigotes was based on RNA levels detected using differential display methodology (Gamboa et al., 2007); interestingly, OPB was not one of the genes found to have higher expression in *L. major* amastigotes in another differential display analysis (Ouakad et al., 2007). OPB was also not found in a microarray analysis of RNA expressed at differential levels between promastigotes and amastigotes of *L. mexicana* (Holzer et al., 2006). The analysis of *L. donovani* expression levels was completed using proteomic techniques and axenic amastigotes (Rosenzweig et al., 2008). As well as using a different species, *L. major*, I used amastigotes isolated from an infected mouse, which may also account for the difference.

The localisation of OPB to the cytosol by immunofluorescence is consistent with the previous localisation of the OPBs of *T. cruzi*, *T. congolense* and *T. evansi* (Caler et al., 1998; Morty et al., 1999a; Morty et al., 2005a). The release of OPB by *L. major* was not found using the methods of this study, where potential release from amastigotes into macrophages was investigated by immunofluorescence, due to cross-reactivity of the anti-OPB antibody with proteins in the macrophage (Section 4.2.3). However, *T. cruzi* OPB has been found to be released by trypomastigotes (Fernandes et al., 2005) and *L. donovani* OPB by promastigotes (Silverman et al., 2008), both by assaying OPB activity released into growth media. This could indicate a role for OPB in the survival of *Leishmania* promastigotes within the sand fly gut or, if this finding was replicated in amastigotes, then OPB could also be involved in survival in the mammalian host.

This role could be in degradation of host peptides within the parasitophorus vacuole for the benefit of continuing infection, whether through impeding host anti-parasite responses or parasite nutrition. Alternatively, OPB could be secreted due to a role in processing exported proteins.

7.2 Inhibition of OPB

Anti-OPB antibodies have been found to inhibit the activity of OPB, both in this study for *Leishmania* (Section 4.2.5), and previously in studies of *T. cruzi*, *T. brucei* and *T. evansi* (Burleigh et al., 1997; Morty et al., 2001; Morty et al., 2005a). These antibodies are too large to inhibit the active site of the peptidase, since access to the active site is blocked by the pore in the β-propeller region of the protein which only allows the passage of small oligopeptides (Fulop et al., 1998; Polgar, 2002). This suggests that at least one of the polyclonal antibodies is possibly acting by blocking the entrance to the pore. This raises the prospect of, as well as testing inhibitors of OPB chosen for their likelihood to interact with the active site, investigating inhibitors of OPB which act by blocking the entrance to the β-propeller pore. Additionally, regions surrounding the β-propeller could be sites of binding for more specific inhibitors of OPB. A number of peptidases found in mammals are also able to cleave following di-arginine residues, including cathepsins B, L, N and W, neprilysin, dipeptidyl peptidase III, trypsin, furin, numerous proprotein convertases and phytepsin (http://merops.sanger.ac.uk). These other enzymes could thus also be inhibited by inhibitors designed to interact with the di-arginine preference of the OPB active site, and so targeting the opening of the β-propeller may result in more specific inhibition.

The effectiveness of inhibitors of OPB in killing *Leishmania* was investigated, since OPB inhibitors have previously been found to be anti-trypanosomal (Morty et al., 2000). Several drugs currently used to treat sleeping sickness are inhibitors of OPB (Morty et al., 1998), and the OPB inhibitor antipain is an antileishmanial compound (Coombs et al., 1982; Coombs and Baxter, 1984). For this investigation a number of polyamine compounds were used. These were chosen due to the similarity of the side chains of two of the compounds to arginine, the preferred P_1 amino acid of OPB, with the remainder of the compounds intended to be controls. However, when the polyamines were assayed both for their ability to inhibit recombinant *L. major* OPB and to kill *Leishmania* promastigotes, a number

of the non arginine-like side chain compounds had IC_{50} values against recombinant OPB which were as low as those of the compounds with the arginine-like side chains (Section 4.2.6). These compounds are unlikely to be competing for the active site in a substrate-mimicking manner, given the dissimilarity of the compounds without the arginine-like side chains to OPB substrates. It is possible that these compounds could be inhibiting OPB via blocking the pore of the β-propeller, rather than the active site itself, though this possibility requires further investigation.

One of the polyamine compounds with an arginine-like side chain, CMR370, and three of the non arginine-like compounds were reasonable anti-leishmanials, with low micromolar LD_{50} values against promastigotes. CMR370 was the best inhibitor of recombinant OPB and was also an inhibitor of Bz-R-AMC hydrolysis by live promastigotes. CMR370 was also tested for its activity against *L. major* amastigotes. However, it was not possible to determine an LD_{50} for this, since CMR370 led to apparent disintegration of the amastigotes, but not clearance of amastigotes from the macrophages. It had been hoped to use luciferase-expressing *Leishmania* for this testing, as then it would be possible to determine that the amastigotes were definitely killed. However, although it appeared a promising side project, it was not possible to validate the use of luciferase-expressing *Leishmania* lines for the detection of intracellular amastigotes in macrophages. Therefore, the traditional method of manual counting of stained slides had to be used for the evaluation of the potential of CMR370 as an anti-amastigote compound and of the infectivity of *OPB* null mutant lines, as detailed below.

7.3 Evaluation of OPB as a Drug Target

To evaluate the usefulness of OPB as a potential drug target, the effect of altering the levels of expression were investigated, with both over-expressers and null mutants of OPB produced. There was no effect on the growth of promastigotes when OPB was over-expressed, though WT[*OPB*] mutants confirmed that an increased level of OPB led to an increase in the hydrolysis of the peptide substrate Bz-R-AMC (Section 5.2.2). Ectopic expression of the active site mutant OPB in wild-type promastigotes, WT[OPB^{S577G}], led to a decrease in the activity towards

Bz-R-AMC. This is probably explained by the mutated OPB competing for the substrate with the native OPB.

OPB was not essential for promastigote stage parasites of *L. major*, since two null mutant clones were produced (Section 5.2.4). The null mutants had a growth defect compared to wild-type promastigotes, with an initial growth lag and a slower rate of growth in log phase. The null mutants conclusively demonstrated that OPB was responsible for the virtually all of the cleavage of Bz-R-AMC, since there was only approximately 1% of hydrolysis of the substrate with these clones, when compared to the wild-type.

The Δ*opb* null mutant clones had a defect in their ability to survive in macrophages, a promising finding suggesting that they are impaired in their ability to transform to mammalian-infective forms (Section 5.2.4.2). A defect in metacyclogenesis was found, meaning fewer promastigotes than normal were developing into the infective promastigote form. The fact that, despite there being some metacyclic promastigotes present, only low levels of amastigotes survive in macrophages suggests that OPB also has a role in either differentiation to amastigotes or in amastigote survival itself. The latter seems likely, since the numbers of amastigotes fell between days 4 and 6 of infection, time points when the differentiation to amastigotes would be complete. The Δ*opb* null mutant promastigotes also had a defect in inducing lesions on the footpads of BALB/c mice, with a lag in growth of the lesion. This lag in growth is likely to be due to both the defect in metacyclogenesis and also the likely additional survival of amastigotes in macrophages. The additional deficiency in differentiation of metacyclic promastigotes to amastigotes or in survival as amastigotes is also suggested by OPB being found to be upregulated in amastigotes of *L. braziliensis* and in the early stages of differentiation of *L. donovani* to amastigotes (Gamboa et al., 2007; Rosenzweig et al., 2008). In order to ascertain the effect on differentiation to amastigotes, purified metacyclic promastigotes should be used for infection of mice/macrophages. The re-expression of OPB in the Δ*opb* null mutants is also necessary to confirm the correct interpretation of the results using the null mutants. Also, as it is not currently possible to differentiate *L. major* promastigotes to amastigotes axenically, it would be necessary to produce *OPB* null mutants in another species in order to investigate differentiation to amastigotes *in vitro*.

It is likely that there may be more effect of the lack of OPB on lesion development in other mice strains or in the natural host. BALB/c mice, the strain which was used in this study, are particularly susceptible to infection with *Leishmania* (Chen et al., 2005). The use of other, less susceptible, mice strains could be a more natural host for determining the role of OPB. In addition, it may be that initiating infection of mice with numbers of promastigotes closer to those naturally egested upon bloodfeeding of sand flies could lead to a different result. Up to around 1,000 *L. major* promastigotes have been shown to be egested by *Phlebotomus papatasi* (Warburg and Schlein, 1986), whereas 50,000 promastigotes were injected into the BALB/c mice in this study.

Even though inhibitors of OPB can kill *Leishmania*, it is likely that the inhibitors target other peptidases in *Leishmania*, given that Δ*opb* null mutants can survive as both promastigotes and amastigotes. This was found with antipain, which is both an OPB inhibitor and an antileishmanial, when the antileishmanial activity of antipain was tested on Δ*opb* null mutants (Section 5.2.4.2). Antipain was still able to kill these null mutant *L. major*, showing that other targets are involved. The ability to generate null mutants of *OPB* and the ability of these mutants to infect BALB/c mice does mean that it is probably not suitable as a primary drug target, based on the current examination of the null mutants, though it could perhaps be targeted as part of a combinatorial approach. Given that OPB appears to be involved in differentiation to infective parasite forms, this targeting could perhaps include a transmission hindering capacity or as prophylaxis to target differentiation to amastigotes, if OPB is confirmed to be involved in this differentiation. Additionally, if it is found that the secretion of OPB by promastigotes is relevant for survival in sand flies, it could be that treatment of the mammalian host with irreversible OPB inhibitors, though not curing infection in the current host, could reduce the ability of parasites to survive in the sand fly, thus also reducing transmission. Transmission-blocking drugs have been investigated for malaria for several years (Butcher, 1997; Coleman et al., 2001; Ponsa et al., 2003) and could also be useful for leishmaniasis. In addition, as OPB has been suggested to be a potential drug target for American and African trypanosomes (Fernandes et al., 2005; Morty et al., 2005b) and for bacteria, treatments developed for other diseases could be investigated. New antibacterial compounds are urgently needed and are being developed, with OPB being one potential target for anti-Gram-positive bacteria (Coetzer et al., 2008; Yu et al., 2008). A number of

inhibitors of bacterial OPB have been found to successfully kill Gram-positive bacteria (Yu et al., 2008). It is thus possible that drugs which have been developed and tested to treat bacterial diseases or trypanosomes could then be investigated for use as antileishmanials, with a consequently much reduced cost of development.

7.4 Role of OPB

The physiological substrates of OPB in the organisms which possess the peptidase are still in doubt (Coetzer et al., 2008). However, OPB has been suggested to be a virulence factor of both bacteria and trypanosomatids. The OPBs of various species have been found to hydrolyse a number of biologically active peptides rich in basic residues, including atrial natriuretic factor (Troeberg et al., 1996; Bagarozzi et al., 1998; Morty et al., 2005a), adrenocortotropic hormone (de Andrade et al., 1998; Tsuji et al., 2004), angiotensins I and II (Nishikata, 1984; Bagarozzi et al., 1998), neurotensin (Kanatani et al., 1991; Troeberg et al., 1996), serum thymic factor (Nishikata, 1984) and reduced [Arg^8] or [Lys^8] vasopressin (Troeberg et al., 1996). Cleavage of atrial natriuretic factor in animals infected with African trypanosomes has been observed (Ndung'u et al., 1992; Morty et al., 2005a) and shown to be due to OPB: the cleavage of atrial natriuretic factor by *T. evansi* OPB can be inhibited in infected rats using anti-OPB antibodies (Morty et al., 2005a). The physiological relevance of cleavage of the other substrates is unknown. Additionally, paired basic residues are abundant in the regions of processing of pro-peptides to peptides in biologically active molecules (Kreil, 1990), suggesting the possibility that OPB could be involved primarily in processing.

The roles of OPB in bacteria have not been determined (Polgar, 2002), though the enzyme is not essential for growth of *Treponema denticola* (Fenno et al., 2001) nor *Salmonella enterica* (Rea and Fulop, 2006). In plants, the role of OPB similarly has not been fully elucidated, though it is thought to be involved in germination. This has been suggested by study of wheat, *Triticum aestivum*, in which the amount of OPB increased as the wheat embryo germinated, suggesting a role in the processing and turnover of peptides in germination (Tsuji et al., 2004). Another role of the OPB of plants, though outside the plant itself, has been suggested by study of the OPB of the pollen of ragweed, *Ambrosia artemisiifolia*,

which can cleave a number of biologically active peptides involved in maintaining bronchial function, including atrial natriuretic factor (ANF) and angiotensins I and II. It may thus be at least partially responsible for the role of ragweed pollen in stimulating respiratory symptoms in hay fever and asthma sufferers (Bagarozzi et al., 1998).

In trypanosomes the role of OPB has been investigated more fully. OPB of African trypanosomes is released into the bloodstream of infected animals by lysis of dying parasites, where it is not inhibited by plasma serpins or α2-macroblobulin (Troeberg et al., 1996; Morty et al., 2001; Morty et al., 2005a). OPB is thought to be responsible for the observed reduction in the levels of the regulatory peptide hormone ANF in the plasma of *T. brucei*-infected dogs and of *T. evansi*-infected rats (Ndung'u et al., 1992; Morty et al., 2005a), with anti-OPB antibodies leading to dramatically reduced hydrolysis of ANF in the blood of *T. evansi*-infected rats (Morty et al., 2005a). The hydrolysis of ANF could result in the loss of control of blood volume and circulatory system lesions observed in trypanosome infections (Coetzer et al., 2008). The ability of inhibitors of OPB to improve the survival rate of mice infected with *T. brucei* gives support for a role in the pathology of trypanosomiasis (Morty et al., 2000). As there was no significant correlation between the inhibition of OPB and the *in vitro* antitrypanosomal effect of these inhibitors, it has been proposed that they were actually mainly acting on the OPB in the host serum, thus influencing the course of the infection (Morty et al., 2000).

In *T. cruzi*, OPB is involved in the invasion of non-phagocytic cells by trypomastigotes (Burleigh and Andrews, 1995; Burleigh et al., 1997; Caler et al., 1998), by triggering an increase in the free intracellular calcium concentration. TcOPB does not signal directly for Ca^{2+} release, but is responsible for generating a Ca^{2+}-signalling factor, which is exported from the trypomastigote, binds a host cell receptor and leads to the release of Ca^{2+} from intracellular stores (Burleigh et al., 1997). The Ca^{2+}-signalling activity was inhibited by OPB inhibitors and specific anti-*T. cruzi* OPB antibodies (Burleigh et al., 1997). *OPB* null mutant *T. cruzi* trypomastigotes are 75% less infective than wild-type and the parasitaemia in mice was reduced when heterozygote or *OPB* null trypomastigotes were used as the infectants (Caler et al., 1998). *OPB* null mutant parasites could not induce Ca^{2+} transients and as the Ca^{2+}-signalling activity was restored by adding recombinant OPB, the central role of OPB in *T. cruzi* pathogenesis was confirmed (Caler et al., 1998). However, OPB has also been found to be released by trypomastigotes, so

instead of cleaving an intracellular factor which is released to bind to the host cell, OPB could be cleaving a factor on the external surface of the parasite to enable uptake of *T. cruzi* (Fernandes et al., 2005). Alternatively, it could interfere with host peptides or physiology, in a similar manner to that postulated for OPB in African trypanosome infections.

As mentioned above, in *Leishmania* the role of OPB may be in the sand fly host, as it has been shown to be released from promastigotes (Silverman et al., 2008). It is possible that OPB is not essential for the growth of promastigotes in axenic culture conditions, but may have a role in survival in the sand fly gut, whether through degradation of peptides in the gut for nutrition or avoidance of host defence mechanisms. Antimicrobial peptides (AMPs) are an important part of the immune response of the insect gut against both bacterial and parasitic pathogens (Boulanger et al., 2004). A variety of AMPs are thought to be present in sand flies, with a screen of a cDNA library from *Lutzomyia longipalpis* identifying 43 immunity-related genes (Dillon et al., 2006). Although many of the AMPs of sand flies are yet to be determined, one, a hemolymph peptide of the insect defensin family, has been shown to be upregulated upon infection of *Phlebotomus duboscqi* sand flies with *L. major* (Boulanger et al., 2004). It is possible that OPB secreted from promastigotes in the sand fly could be interfering with sand fly AMPs as a method of evading defence mechanisms.

Investigation of whether amastigotes also secrete OPB is also a necessary step in the determination of its role in their survival in mammalian hosts. The low survival of the null mutant clone amastigotes within macrophages found in this study, despite the presence of some metacyclic promastigotes, suggests that OPB may have a role in the survival of amastigotes within macrophages. This role could potentially be linked to the secretion of OPB, with OPB potentially being involved in the degradation of host peptides in the parasitophorous vacuole.

Alternatively, *Leishmania* OPB has been suggested to influence infection of macrophages through the activation of leishporins, which are cytosolic proteins which can damage macrophage membranes by forming transmembrane pores. They are secreted by the parasite and are thought to generate pores in either the parasitophorous vacuole membrane and/or the macrophage cell membrane, facilitating the release of amastigotes (Almeida-Campos and Horta, 2000). OPB possibly releases the active leishporin by hydrolysing an oligopeptide inhibitor,

though the activity was only generated by *L. amazonensis* cytosolic extract, and not *L. guyanensis* cytosolic extract (Almeida-Campos and Horta, 2000). Whilst OPB from *L. guyanensis* has not been reported, OPB has been documented in the genome of the closely related species *L. braziliensis* (Peacock et al., 2007). As OPB has been found in all trypanosomatids so far examined, it seems unlikely to have been completely lost from *L. guyanensis*. However, as the likely OPB reported by de Andrade et al. from *L. amazonensis* (de Andrade et al., 1998) could activate the leishporin, it suggests that OPB is at least capable of this activation, though it may not be physiologically relevant. If activation of leishporin was reported with *L. major* cytosolic extract, the role of OPB could be tested using the Δ*opb* null mutants. Investigation of the role of OPB would also require confirmation of the ability of the amastigote to secrete OPB, in order to activate the leishporin in the parasitophorous vacuole.

The role of OPB in metacyclogenesis, or differentiation to amastigotes, is unlikely to be linked to secretion of the peptidase. The role could be akin to the defect in metacyclogenesis caused by a lack of autophagy (Besteiro et al., 2006), a self-digestion process whereby cells degrade proteins and organelles in the cytoplasm, by sequestration into a double-membrane vesicle, which normally fuses with the lysosome to allow the degradation of the cargo by hydrolases (Reggiori and Klionsky, 2005). It is possible that OPB aids metacyclogenesis or differentiation through degradation of peptides in the cytosol, providing material for the remodelling of the parasite undertaken at this time, analogous to the degradation of proteins in the lysosome through autophagy. Alternatively, OPB could be involved in the processing of propeptides or other regulatory peptides involved in a cascade leading to differentiation to infective parasite forms.

7.5 Conclusions and Future Directions

The detection of peptidase activity in live *Leishmania* using the fluorogenic substrate Bz-R-AMC has proved a fruitful line of study, enabling the characterisation of the activity of the serine peptidase OPB in live *L. major* promastigotes. OPB is expressed in similar levels in all three main stages of the life cycle of *L. major* and also in other *Leishmania* species. The inhibition profile of OPB was determined in live promastigotes and OPB was localised to the cytosol, the site of Bz-R-AMC hydrolysis. Immunoprecipitation confirmed that OPB was

responsible for the cleavage of Bz-R-AMC, with anti-OPB antibodies inhibiting the hydrolysis. Inhibitors of OPB also killed *Leishmania* promastigotes.

Genetic manipulation of OPB was successfully completed, with both mutants over-expressing OPB and Δ*opb* null mutants produced. Thus, OPB was found not to be essential for the growth of promastigote *L. major*, though lack of OPB did lead to a defect in metacyclogenesis, a defect in the ability to survive in macrophages and a reduced ability to induce lesions on the footpads of BALB/c mice.

A number of areas for future research are suggested by the findings of this study. Crucially, re-expression of OPB in the Δ*opb* null mutants is necessary, in order to confirm the phenotypic deficiencies of the null mutants are due to the lack of OPB.

Since it appears that OPB may have a role in either differentiation to amastigotes and/or in amastigote survival itself, over and above the role of OPB in metacyclogenesis, a full investigation of its involvement in these processes should be undertaken. To investigate the amastigote-specific effects, purified metacyclic promastigotes could be used to infect mice and macrophages. This would determine whether or not OPB indeed is important for differentiation to the amastigote form and/or amastigote survival.

Additionally, confirmation of the release of OPB by promastigotes and investigation of its release by amastigotes are required. Secretion by promastigotes suggests a role in parasite survival in the sand fly or differentiation through the various developmental stages that occur there; it would be interesting to know how important OPB is for completing the life cycle in the insect vector and in transmission to a mammalian hosts. Release of OPB from amastigotes would suggest that OPB also plays some role in the parasite's interaction with the macrophage.

Finally, a crucial area for the future study of OPB is to establish the physiological substrate(s), as this would greatly aid identification of the role of OPB. This may be possible using the Δ*opb* null mutants generated in this study, perhaps by proteomic comparison with wild-type parasites. Alternatively, immunoprecipitation of substrates could be attempted. Discovering the role of OPB could also lead to further understanding of the mechanisms of differentiation of *Leishmania*.

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