

Antileishmanial drugs: search for SIR2 inhibitors

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Author's Declaration

Under the terms of the Decree-Law nº 216/92, of October 13th, is hereby declared that the following original articles were prepared in the scope of this dissertation.

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* Authors have equally contributed to the work.

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Under the terms of the referred Decree-Law, the author declares that he afforded a major contribution to the conceptual design and technical execution of the work, interpretation of the results and manuscript preparation of the published articles included in this dissertation.



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Summary

Leishmaniasis is a parasitic disease, caused by the protozoa of the genus *Leishmania* that affects millions of people worldwide, especially in tropical and subtropical areas, and is responsible for high mortality and morbidity. Disease control is dependent on drug therapy, since no approved human vaccine is available. However, the existing therapy is far from satisfactory owing to the emergence of resistances, toxicity and its limited efficacy due to disease exacerbation, mainly associated with compromised immune capability.

The proteins belonging to the Silent Information Regulator 2 (SIR2) family, also known as Sirtuins are conserved throughout evolution and are classified as class III histone deacetylases (HDAC) due to their dependency on NAD^+ to deacetylate lysine residues of histones and non-histone substrates. Moreover, these proteins have been involved in the regulation of a number of biological processes such as gene silencing, DNA repair, longevity, metabolism, apoptosis, and development. One of the three SIR2 homologues identified in the *Leishmania* genome, the SIR2RP1, is localized in the cytosol and, according to indirect molecular as well as biological approaches, seems to be involved in parasite survival. A genetic approach through the disruption of the *Leishmania* SIR2RP1 encoding gene suggested that this protein was determinant to parasite survival due to the impossibility of generating null chromosomal mutants without episomal rescue. Furthermore, disruption of one LiSIR2RP1 gene allele (LiSIR2^{+/-}) led to decreased amastigote virulence, *in vitro* as well as *in vivo*. Biochemical approaches revealed that LiSIR2RP1 has NAD^+ -dependent deacetylase and ADP-ribosyltransferase activities. Moreover, we found that LiSIR2RP1 is partially associated with the parasite's cytoskeleton and is capable of deacetylating tubulin, either in dimers or, when present, in taxol-stabilized microtubules or in promastigote and amastigote extracts, which may have significant implications during the remodelling of the parasite's morphology and its interaction with the host cell. These findings led us to consider LiSIR2RP1 as a potential therapeutic target. Therefore, aiming at the identification of LiSIR2RP1 inhibitors, an "*in silico*" screening of the National Cancer Institute compounds 3D database (NCI-2000) containing approximately 2×10^5 compounds was conducted, seeking the inhibition of the parasite SIR2 over the human enzyme, SIRT1. Even though this strategy was effective in identifying N-(2-

fluorophenyl) nicotinamide, which can be used for lead designing, it failed to identify a truly potent and selective lead compound.

The presence of a common structural moiety (naphthalene) in some molecules identified as Sirtuin inhibitors, led us to discover bisnaphthalimidopropyl (BNIP) derivatives as a new class of inhibitors. Structure-activity studies were conducted with 12 BNIP derivatives leading to the identification of BNIPDanonane as a potent and selective inhibitor of the LiSIR2RP1 versus its human counterpart. Indeed, based on kinetic studies, its inhibitory effect is due to competition with NAD^+ , which is supported by docking analysis of BNIP derivatives in the LiSIR2RP1 and hSIRT1 three-dimensional models. Furthermore all the BNIP derivatives are active *in vitro* against *Leishmania infantum* intracellular amastigotes (IC_{50} lower than $12\mu\text{M}$) and at least one of them, BNIPDaooctane, was identified to be active *in vivo* since its administration reduces the parasite load in the spleen and liver of a visceral leishmaniasis mice model. Therefore, this work will open new perspectives towards the development of LiSIR2RP1 inhibitors, which interfere with the parasite development.

Résumé

La leishmaniose est une maladie parasitaire, causées par des protozoaires du genre *Leishmania*, qui affecte des millions de personnes dans le monde, en particulier dans les régions tropicales et subtropicales, et est responsable de mortalité et de morbidité importante. La lutte contre cette maladie dépend de la chimiothérapie, car aucun vaccin pour l'homme approuvé n'est disponible. Toutefois, la thérapie existante est loin d'être satisfaisante en raison de l'émergence de résistances et de la toxicité relativement importante des médicaments utilisés.

Les protéines appartenant à la famille SIR2 (Silent Information Regulator 2) également connues sous le nom de Sirtuins sont conservées tout au long de l'évolution et sont regroupées dans la classe III des Histones Déacetylases (HDAC), en raison de leur dépendance du co-facteur NAD^+ pour l'expression de leur activité enzymatique vis-à-vis des résidus lysine de substrats histones et non-histones. En outre, ces protéines ont été impliquées dans de nombreuses fonctions biologiques comme la répression transcriptionnelle «silencing», la réparation de l'ADN, la longévité, le métabolisme, l'apoptose et le développement. L'une des trois protéines homologues de SIR2 identifiées dans le génome de *Leishmania*, c'est-à-dire la SIR2RP1, est localisée dans le cytosol et semble être impliquée dans la survie du parasite. En effet, les techniques de génétique inverse ont permis de démontrer que le gène était essentiel pour la survie des formes amastigotes. En effet, il a été impossible de générer des mutants nul pour le gène *LiSIR2RP1* sans complémentation avec un plasmide ectopique portant le gène *LiSIR2RP1* «episomal rescue». En revanche, l'inactivation d'un allèle *LiSIR2RP1* conduit à l'obtention d'amastigotes simples mutants ($\text{LiSIR2RP1}^{+/-}$) dont la virulence *in vitro* et *in vivo* est atténuée. Les approches biochimiques ont montré que cette protéine avait une dualité fonctionnelle exprimant deux activités enzymatiques: désacétylase NAD^+ -dépendante et ADP-ribosyltransférase. En outre, nous avons constaté que LiSIR2RP1 est en partie associée au cytosquelette et est capable de désacétyler la tubuline qu'elle soit sous forme de dimères ou présente dans les microtubules stabilisés par le taxol ou dans les extraits de promastigotes et amastigotes. Ces observations peuvent avoir une implication dans le remodelage du cytosquelette au cours de la différenciation du parasite. Ces résultats nous ont amenés à examiner si LiSIR2RP1 pouvait être une cible thérapeutique. En se basant sur la structure cristallographique de la SIRT1 humaine, le site actif de LmSIR2RP1 (SIR2RP1) de *L.*

major été modélisé. Le criblage virtuel d'une chimiothèque du [National Cancer Institute 3D database (NCI-2000)] qui contient approximativement 2×10^5 molécules a été réalisé en se basant sur l'empreinte du nicotinamide. Le docking *in silico* nous a permis de retenir 11 composés qui ont été testés *in vitro* vis-à-vis des parasites et de l'activité enzymatique désacétylase NAD⁺dépendante présente dans un extrait parasitaire. Un des composés: le N-(2-fluorophényl) nicotinamide a montré une activité inhibitrice significative de la croissance des parasites *in vitro* et de l'activité enzymatique désacétylase NAD⁺-dépendante présente dans un extrait parasitaire ce qui est une preuve du concept. Cependant, d'autres modifications sont nécessaires pour améliorer la performance de ce composé.

La présence d'une structure commune (naphtalène) dans certaines molécules inhibitrices de l'activité des Sirtuin nous a amené à explorer l'effet de composés bisnaphthalimidopropyl (BNIP) qui comportent ce type de structure sur l'activité enzymatique de LiSIR2RP1. Nous avons pu identifier une molécule: le BNIPDanonane comme un puissant inhibiteur sélectif et de la LiSIR2RP1 par rapport à son orthologue humain. Les analyses de modélisation ont pu montrer que l'activité inhibitrice résulte vraisemblablement d'une compétition avec le NAD⁺. Par ailleurs, toutes les molécules BNIP sont inhibitrices *in vitro* de la croissance des amastigotes intracellulaires de *Leishmania infantum* (IC₅₀ inférieur à 12 μ M) et au moins une des molécules le BNIPDaooctane, a montré une activité *in vivo* puisque son administration aux souris infectées réduit la charge parasitaire dans la rate et le foie. Ces travaux ouvrent des perspectives pour le développement de drogues inhibitrices de LiSIR2RP1 qui interfèrent avec le développement parasitaire.

Resumo

A leishmaniose é uma doença parasitária causada pelo protozoário do género *Leishmania*, que afecta milhões de indivíduos, em particular nas regiões tropicais e subtropicais, sendo deste modo responsável por uma elevada mortalidade e morbilidade. Na ausência de vacinas para uso humano, o controlo da doença baseia-se na terapêutica farmacológica. No entanto, os medicamentos disponíveis não são satisfatórios principalmente devido ao aparecimento de resistências, aos efeitos laterais indesejáveis, e sobretudo devido à sua eficácia ser limitada em situações de exacerbação da doença, como a que ocorre em indivíduos com o sistema imunológico comprometido.

As proteínas pertencentes à família "Silent Information Regulator 2" (SIR2), também designadas de Sirtuins, são proteínas conservadas ao longo da evolução e classificadas como histonas desacetilases (HDAC) da class III, uma vez que dependem do NAD^+ como co-factor para desacetilar os resíduos de lisina de diversos substratos. As proteínas incluídas nesta família têm-se revelado envolvidas na regulação de vários processos biológicos como o silenciamento de genes, reparação do DNA, longevidade, metabolismo, apoptose e desenvolvimento. No genoma da *Leishmania* foi identificada a existência de informação genética para codificar três proteínas homólogas às da família SIR2. Uma das proteínas, designada de SIR2RP1, encontra-se localizada no citosol e de acordo com uma abordagem molecular e biológica indirecta parece estar envolvida na sobrevivência do parasita. A impossibilidade de conseguir remover os dois alelos que codificam para esta proteína em *Leishmania*, sem ter havido suplemento episomal, sugere um envolvimento determinante da mesma na sobrevivência do parasita. Para além do que, a remoção de apenas um alelo que codifica a proteína LiSIR2RP1 compromete virulência do parasita tanto *in vitro* como *in vivo*. A caracterização bioquímica revelou que a proteína LiSIR2RP1 apresenta actividade desacetilase e ADP-ribosiltransferase dependente do NAD^+ . Adicionalmente, observamos que esta proteína se encontra parcialmente associada ao citoesqueleto do parasita catalizando a desacetilação da tubulina na presença de NAD^+ . Com base nestes resultados, consideramos a possibilidade de a proteína LiSIR2RP1 representar um potencial alvo terapêutico. Com o objectivo de identificar novas moléculas que inibam a proteína SIR2 do parasita e não a proteína humana correspondente, SIRT1, procedeu-se posteriormente ao rastreio "*in silico*" da livreria de compostos

do “National Cancer Institute 3D database (NCI-2000)” contendo aproximadamente 2×10^5 compostos. Contudo, esta estratégia não permitiu a identificação de um inibidor potente, permitindo apenas a identificação do N-(2-fluorfenil)nicotinamida, que poderá ser posteriormente submetido a modificações químicas. Avaliamos também a capacidade dos compostos derivados do bisnaftalimidopropil (BNIP) inibirem a proteína LiSIR2RP1. A realização de um estudo de relação estrutura-actividade, que incluiu 12 derivados do BNIP, a proteína LiSIR2RP1, e a proteína humana correspondente, permitiu identificar os derivados BNIP como uma nova classe de inibidores das Sirtuins. O composto BNIPDanonano foi seleccionado como um inibidor potente e selectivo da enzima LiSIR2RP1. Pela realização de cinéticas enzimáticas e análise computacional de modelos 3D das proteínas, concluímos tratar-se de um inibidor que actua por competição com o NAD^+ . Verificamos também que todos os compostos derivados do BNIP inibem, em ensaios *in vitro*, o crescimento da forma amastigota intracelular de *L. infantum* (IC_{50} inferiores a $12 \mu\text{M}$). Pelo menos o derivado BNIPDaotano foi eficaz a reduzir a carga parasitária no fígado e baço de ratinhos infectados. Este estudo abre novas perspectivas ao desenvolvimento de inibidores da proteína LiSIR2RP1, que interfiram com o crescimento do parasita.

Abbreviations list

ACR2	antimonite reductase 2
AceCoA	acetyl-coenzyme A
AceCS	acetyl-coenzyme synthase
aP2	fatty acid binding protein
APC	antigen presenting cells
BNIP	bisnaphthalimidopropyl
Cdks	cyclin-dependent kinases
CD	clusters of differentiation
CL	cutaneous leishmaniasis
CR1 or CR3	complement receptor 1 or 3
CRP	C-reactive protein
CR	caloric restriction
DAT	direct agglutination test
DC	dendritic cells
DCL	diffuse cutaneous leishmaniasis
DC-SIGN	dendritic cell-specific intracellular adhesion molecule 3 grabbing nonintegrin
DNA	deoxyribonucleic acid
DDT	dichloro-diphenyl-trichloroetthane
ERK	extracellular regulated kinase
FACS	fluorescence-activated-cell-sorter
FDA	Food and Drug Administration
FAST	fast agglutination screening test
FML	fucose-mannose-ligand
FOXO	forkhead box class O
GDH	glutamate dehydrogenase
GSH	reduced glutathione
GSSG	oxidized glutathione
GIPL	glycosylinositolphospholipids
HAT	histone acetyltransferases
HDAC	histone deacetylases
HIV	human immunodeficiency virus

HSP	heat shock protein
IFN	interferon
Ig	immunoglobulin
IL	interleukin
i.p.	intraperitoneal
i.v.	intravenous
IMPDH	inosine 5'-monophosphate dehydrogenase
iNOS	inducible nitric oxide synthase
IGF	insulin like growth factor
JNK	Jun n-terminal kinase
kDNA	kinetoplast DNA
LACK	<i>Leishmania</i> analogue to receptors for mammalian kinase c
LiSIR2+/-	<i>Leishmania infantum</i> silent information regulator 2 single knockout
LiSIR2RP1	<i>Leishmania infantum</i> Silent Information Regulator 2 related protein 1
LPG	lipophosphoglycan
LPS	lipopolysaccharide
MAP	mitogen activated protein
MAPK	mitogen activated protein kinase
MCL	mucocutaneous leishmaniasis
MESP	molecular electrostatic potentials
MFR	mannose-fucose receptor
MHC	major histocompatibility complex
MRP	multi-drug resistance related protein
MyoD	myogenic differentiation
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
Nampt	nicotinamide phosphoribosyltransferase
NF- κ B	nuclear factor kappa-B
NK	natural killer
NO	nitric oxide
OAADPr	O-acetyl-ADP-ribose
PARP	polymerase ADP-ribosyltransferase
PCD	programmed cell death
PCR	polymerase chain reaction
PKC	protein kinase C
PKDL	post kala-azar dermal leishmaniasis

PS	phosphatidylserine
PSG	promastigotes secretory gel
PTPase	protein tyrosine phosphatase
PV	parasitophorous vacuole
RNA	ribonucleic acid
RNAi	rNA interference
ROS	reactive oxygen species
SbIII	trivalent antimonial
SbV	pentavalent antimonial
SHP-1	protein tyrosine phosphatase
SIR2	Silent information regulator 2
SIRT	Human silent information regulator
Th	helper T lymphocytes
TGF	transforming growth factor
TLR	tool-like receptor
TNF	tumour necrosis factor
TR	trypanothione reductase
Treg	tegulatory T cells
TS ₂	oxidized trypanothione
TSA	thiol-specific antioxidant
T(SH) ₂	reduced trypanothione
VL	visceral leishmaniasis
WHO	World Health Organization
WT	wild type

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Chapter I

Leishmania spp. and leishmaniasis

1. The *Leishmania* parasite

1.1 History, taxonomy and clinical manifestations

In 1903, two independent reports from William Leishman and Charles Donovan described the protozoan parasite known as *Leishmania donovani* in the spleen aspirates from patients in India afflicted with the life-threatening disease nowadays called visceral leishmaniasis.

Since then, more than 20 *Leishmania* species and subspecies with the ability to cause the disease in humans have been described (Ashford et al., 2000). They belong to the *Trypanosomatidae* family in the *Kinetoplastidae* order, which is mainly characterized by the presence of a kinetoplast in their members. Indeed, the protozoan parasites of the genus *Leishmania* are the causative agents of leishmaniasis, whose broad spectrum of clinical manifestations reflects the heterogeneity between the *Leishmania* species (Table I). Thus, leishmaniasis is classified as (Chappuis et al., 2007):

Cutaneous leishmaniasis (CL): emergence of one or several ulcer(s) or nodule(s) in the exposed areas of the skin. The ulcers usually self-heal in immunocompetent individuals, but leave disfiguring scars (Figure 1A).

Mucocutaneous leishmaniasis (MCL): progressively destructive ulcerations of the mucosa, extending from the nose and mouth to the throat cavities and surrounding tissues, also known as espundia. These lesions are not self-healing and are difficult to treat (Figure 1B).

Visceral leishmaniasis (VL): systemic disease also known as kala-azar, which is fatal when left untreated. High fever, substantial weight loss, anaemia, and swelling of the liver and spleen are the main symptoms of this disease (Figure 1C).

Post-kala-azar dermal leishmaniasis (PKDL): complication of visceral leishmaniasis frequently observed after treatment, and characterized by a macular, maculo-papular or nodular rash (Figure 1D).

Nevertheless, all forms of this protozoan infection share three pathogenic features: resident tissue macrophages are targeted and support intracellular parasite multiplication; the host's immunoinflammatory response regulates the outcome of the disease, and persistent tissue infection is characteristic (Murray et al., 2005).



Figure 1. Clinical manifestations of leishmaniasis. Patients with: cutaneous leishmaniasis (A), mucocutaneous leishmaniasis (B), visceral leishmaniasis (C) and post-kala-azar dermal leishmaniasis (D) (Adapted from Chappuis et al., 2007).

Table I. Principal *Leishmania* species and their geographical distribution

Species ^a	Pathology	Reservoir	Distribution
<i>L. (L.) donovani</i>	VL; PKDL	Human	Africa; India (Old World)
<i>L. (L.) infantum</i>	VL; CL(rare)	Dog	Mediterranean; Asia and sub-Saharan (Old World)
<i>L.(L.) chagasi</i> ^b	VL; CL(rare)	Dog	Latin America (New World)
<i>L.(L.) tropica</i>	CL; VL (rare)	Human	Middle East, India, Mediterranean, Western Asia (Old World)
<i>L. (L.) major</i>	CL	Rodents	Middle East, China, India, Pakistan, Africa (Old World)
<i>L.(L.) aethiopica</i>	CL; DCL	Rodents	East Africa (Old World)
<i>L. (L.) mexicana</i>	CL; DCL	Rodents	Central America (New World)
<i>L. (L.)amazonensis</i>	CL; DCL; MCL; VL (rare)	Rodents	Central and South America (New World)
<i>L. (V.) braziliensis</i>	CL; MCL	Rodents; Sloth; Opossum	South America (New World)
<i>L.(V.) guyanensis</i>	CL	Sloth	South America (New World)
<i>L.(V.) panamensis</i>	CL	Sloth	South America (New World)
<i>L.(V.) peruviana</i>	CL	Human; Dog (?)	Central and South America (New World)

VL, visceral leishmaniasis; PKDL, post kala-azar dermal leishmaniasis; CL, cutaneous leishmaniasis;

DCL, diffuse cutaneous leishmaniasis; MCL, mucocutaneous leishmaniasis;

^a Both subgenera *Leishmania* *Leishmania* *L.(L.)* and *Leishmania* *Viania* *L.(V.)* are indicated;

^b Growing evidences that *L. (L.) chagasi* and *L. (L.) infantum* are the same specie;

1.2 The life cycle

Leishmania parasites have a digenetic life cycle displaying two main developmental stages through their life-cycle: an insect vector stage and a vertebrate host stage (Figure 2). Indeed, two main morphological forms of the parasite can be

distinguished: a slender flagellated promastigote form that replicates in the insect vector digestive tract, and a round or oval aflagellated non-motile amastigote form that resides and replicates inside the phagolysosomal vacuoles of the host mononuclear phagocyte cells (Figure 3A and B respectively) (Solbach and Laskay, 2000). However, other cell types like fibroblasts and dendritic cells may also harbor the parasite (Moll et al., 1995; Hervas Rodriguez et al., 1996).

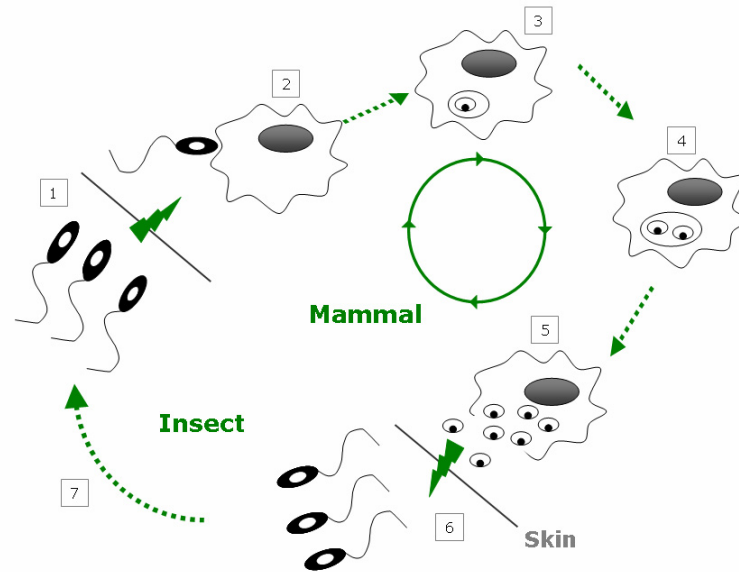


Figure 2. *Leishmania* life cycle. *Leishmania* metacyclic promastigotes are delivered to a vertebrate host by the bite of an infected sandfly (1). Promastigotes attach to phagocytic cells and are readily engulfed (2). Within the phagolysosome, promastigotes differentiate into amastigotes (3) that replicate (4) and are released from the infected host cells (5) spreading the infection into the vertebrate host. When a sandfly ingests a blood meal from an infected host, the amastigotes differentiate back into promastigotes (6) and become metacyclic (7) (Adapted from Ponte-Sucre, 2003).

A vertebrate host becomes infected with *Leishmania* after the bite of an infected Phlebotomine female sandfly when it takes a blood meal, by the injection of metacyclic promastigotes. These non-dividing and infective forms are present on the salivary glands of the sandfly vector and display increased resistance to host complement-mediated cell lysis (Sacks et al., 1992). Indeed, once injected into the vertebrate host, the metacyclic promastigotes are engulfed by the resident dermal or recruited phagocytic cells. Then, the parasite-containing phagosome fuses with a lysosome forming a phagolysosome within which the promastigotes differentiate into the parasite vertebrate stage, the amastigote form. Contrarily to other pathogens that are destroyed by the hostile environment of the phagolysosome, the *Leishmania* parasites are not only resistant to the acidic pH, hydrolytic enzymes

and oxygen and nitrogen intermediates present therein but are also capable of multiplying, leading to the host cell lysis and release of amastigotes that can infect new cells. When a sandfly vector takes a blood meal from an infected vertebrate host, it ingests either free *Leishmania* amastigotes or amastigote-infected mononuclear cells. Within the insect midgut, the amastigotes transform into the flagellated procyclic promastigotes that multiply by binary fission. Some of this procyclic promastigotes differentiate into metacyclic promastigotes (metacyclogenesis) that migrate to the sandfly's salivary glands due to structural modifications of the surface lipophosphoglycan (LPG) (Pimenta et al., 1992; McConville et al., 1992). The infective promastigotes are now ready to be transmitted to a vertebrate host.

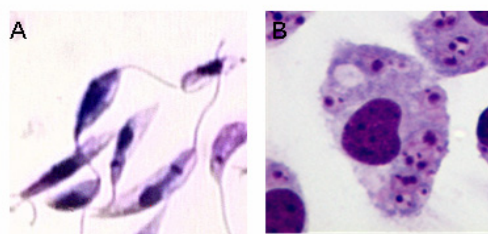


Figure 3. *Leishmania* parasite forms. *Leishmania* promastigotes (A) (Adapted from F. Opperdoes, <http://www.icp.ucl.ac.be>). Wright staining of *Leishmania infantum* intracellular amastigotes in mouse peritoneal macrophages (B) (Adapted from J. Tavares, unpublished data).

1.2.1 Axenic cultures

Several culture mediums have been described as appropriate to the growth of the promastigote form of *Leishmania* (Hendricks et al., 1978; Hart and Coombs, 1981; Sadigursky and Brodskyn, 1986; Lemesre et al., 1988). Indeed, the promastigotes' metacyclogenesis can be mimicked *in vitro*, since procyclic forms correspond to promastigotes in the exponential phase of their growth, and metacyclic forms are found in stationary phase cultures (Sacks and Perkins, 1985). As stationary phase promastigote cultures represent a heterogeneous population, techniques to purify metacyclic promastigotes, based on the structural modifications of LPG, have been described for the several *Leishmania* species. Through the use of procyclic LPG-specific antibodies, it is possible to purify metacyclic promastigotes of *L. donovani* (Sacks et al., 1995), *L. major* (da Silva and Sacks, 1987), *L. amazonensis* (Courret et al., 1999) and *L. tropica* (Lira et al., 1998). The purification of *L. braziliensis* (Pinto-da-Silva et al., 2002) and *L. chagasi* (Soares et al., 2002) metacyclic promastigotes was described using lectin.

The amastigotes can be purified from either experimentally infected animals or from *in vitro* infected host cells, although a limitation concerning the number of parasites isolated has led to the development of axenic culture conditions for amastigotes. The morphological, biochemical, biological and immunological properties of the axenic amastigotes resemble the amastigotes isolated from host infected cells, thus representing an *in vitro* model to *Leishmania* differentiation, immunological and drug research studies (Hodgkinson and Soong, 1997). The experimental conditions for obtaining *L. infantum* axenic amastigotes have been clearly defined by Sereno and Lemesre, 1997.

1.3 Unusual biological characteristics of Trypanosomatids

Trypanosomatid parasites emerge from the most ancient eukaryotic lineages, with branches deeper than those from the younger metazoan kingdoms of fungi, plants and animals (Beverley, 1996). Despite their different life cycles, *Leishmania* spp. and *Trypanosoma* spp. belong to the *Kinetoplastidae* order in the *Trypanosomatidae* family because they have two different genomes: a nuclear and a mitochondrial (kinetoplast).

Moreover, the compartmentalisation of energy metabolism with the glycolytic pathway and other enzymes sequestered in glycosomes, a redox metabolism based on a unique thiol called trypanothione, and a polycistronic transcription with post-translational regulation of gene expression are among the unusual characteristics of trypanosomatids.

1.3.1 Nuclear and kinetoplast genomes

The kinetoplastid parasite's genome comprises the nuclear and the mitochondrial genomes. The *Leishmania* karyotype is conserved among the species but differs in number. Indeed, the Old World *Leishmania* species have 36 chromosomes, compared with 35 from New World species and 34 from the *L. mexicana* complex (Wincker et al., 1996; Britto et al., 1998). Sequencing the genome of three kinetoplastid parasites, *L. major*, *T. brucei* (the agent of African trypanosomiasis) and *T. cruzi* (the agent of Chagas disease), revealed the preservation of a large-scale gene synteny over 200-500 million of years (Ivens et al., 2005; Berriman et al., 2005; El-Sayed et al., 2005; El-Sayed et al., 2005a). The genome sequencing project confirmed, as expected, that the *Leishmania* spp. genome is predominantly diploid. Indeed, *L. major* was the first *Leishmania* spp. whose genome was sequenced (Ivens et al., 2005). Moreover sequences of *L. infantum* and *L. braziliensis'* nuclear genome became recently available (<http://www.sanger.ac.uk/Projects> or <http://www.GeneDB.org>). A comparison of

the three genomes identified only 200 genes mainly involved in host-parasite interactions and parasite survival in macrophage, with differential distribution between the three species (Peacock et al., 2007). The *Leishmania* spp. nuclear genome is known for its plasticity mainly due to pseudogene formation and gene loss (Peacock et al., 2007). Furthermore, *Leishmania* spp. carries additional genetic material on multi-copy minichromosomes that result from amplifications of genomic sequences. Even the precise mechanism is still unknown; their occurrence can be spontaneous or as a consequence of parasite exposure to adverse conditions such as drug selection (Beverley, 1991; Segovia, 1994).

Kinetoplastid parasites have only one giant mitochondria containing one single kinetoplast DNA (kDNA), which is organized in maxi and minicircles. There are a few dozen maxicircles (from 35 to 50 Kb), present in 10-30 copies per cell and which gene products are similar to those of mitochondrial in higher eukaryotes such as respiratory chain components and ribosomal RNA (Liu et al., 2005). Also, there are a few thousand minicircles, which range in size from 0.8 to 1.6 kb and are present at about 30 000-50 000 copies per cell. The minicircles contain genes for guide RNA, needed for editing the cryptic maxicircle transcripts into functional mRNAs (Simpson and Kretzer, 1997).

1.3.2 Gene organization and transcription

Little is known about how transcription is initiated in trypanosomatids. Their unusual chromosome organization in directional gene clusters previously reported to *L. major* and *T. brucei*, became fully evident with entire genome sequencing (Myler et al., 1999; Worthey et al., 2003; El-Sayed et al., 2003; Ivens et al., 2005). In the absence of promoters, RNA polymerase II initiates upstream transcription of most 5' genes of each cluster, originating polycistronic transcripts (Martinez-Calvillo et al., 2004). Thus, *Leishmania* spp. mRNAs are transcribed non-specifically as a polycistronic message that does not appear to encode any functional related proteins. The polycistronic transcript is then processed by *trans*-splicing. All mRNA have a 39bp sequence in common at their 5' end, called spliced leader. Tans-splicing processing cleaves the individual mRNA from their precursor by adding the spliced leader (cleaved from another transcript) to the 5' end and polyadenylating the 3' end (LeBowitz et al., 1993; Ullu et al., 1993; Clayton, 2002). Moreover, the regulation of gene expression is not presumably done at the level of transcription initiation as the rate of polycistronic transcription seems to be stable (Clayton, 2002). Even though the mechanisms of how kinetoplastid parasites regulate gene expression are not fully understood, this appears to involve: the

gene's number of copies, as abundant proteins are commonly found as multi-copy genes, and the post-transcriptional regulation at the level of mRNA stability and targeted mRNA degradation (Clayton, 2002; Campbell et al., 2003).

1.3.3 Redox system

Trypanosomatids possess a unique thiol redox metabolism in which the ubiquitous glutathione/glutathione reductase is replaced by trypanothione, a bisglutathionylspermidine conjugate, and the flavoenzyme trypanothione reductase. Trypanothione is a key molecule for the synthesis of DNA precursors, the homeostasis of ascorbate, the detoxification of hydroperoxides, and the sequestration/export of thiol conjugates (Fairlamb et al., 1985; Fairlamb and Cerami, 1992; Krauth-Siegel and Coombs, 1999; Augustyns et al., 2001; Krauth-Siegel and Schimdt, 2002)

Genetic studies have evidenced the essentiality of trypanothione reductase for the survival of several *Leishmania* and *Trypanosoma* species (Kelly et al., 1993; Dumas et al., 1997; Tovar et al., 1998). The biosynthesis of trypanothione ($T(SH)_2$) consists of synthesis of the precursors glutathione and spermidine which are then combined to form $T(SH)_2$. Despite having a similar redox potential, $T(SH)_2$ is much more reactive than glutathione (GSH) (Fairlamb et al., 1992). The difference in the two thiols reactivity is based in the dithiol character of $T(SH)_2$ which is kinetically favoured as it forms intramolecular disulfides (TS_2) compared to GSH that forms intermolecular disulfides (GSSG) (Gilbert, 1990). Moreover, the lower pK values of trypanothione compared to glutathione are coincident with the intracellular pH of the parasites (Moutiez et al., 1994). The trypanothione reductase play an important role in the parasites redox state as it is the responsible of maintaining the trypanothione pool reduced. $T(SH)_2$ can in turn reduce other thiols such as glutathione and ovothiol (Fairlamb and Cerami, 1992). Moreover the reducing capacity of $T(SH)_2$ is strongly enhanced in the presence of tryparedoxins, a small dithiol proteins. Indeed the $T(SH)_2$ /tryparedoxins system catalyse the reduction of hydroperoxides and ribonucleotides (Nogoceke et al., 1997; Dormeyer et al., 2001 Flohe et al., 2002;).

1.4 Vectors and reservoirs

1.4.1 Vectors

Phlebotomine female sandflies are dipteran insects within the family of *Psychodidae* (Figure 4). The transmission of leishmaniasis is attributed to about 70 of around 1000 known sandfly species (Murray et al., 2005). Those belonging to the genus *Lutzomya* are prevalent in the New World (ie, the Americas), and those belonging to the genus *Phlebotomus* are prevalent in the Old World (ie, Africa, Europe and Asia). Phlebotomine sandflies are not active during the day and seek out cool and relatively humid dark niches which allow them to survive in hot and dry climates. Indeed, they become active at dusk when the temperature drops and humidity rises.



Figure 4. *Leishmania* vector: Phlebotomine female sandfly. *Leishmania* infections typically occur through the bite of sandflies belonging to either *Phlebotomus* spp. (Old World) or *Lutzomya* spp. (New World) (Adapted from http://www.who.int/leishmaniasis/disease_epidemiology/en/index.html).

The classification of the mammal-infective *Leishmania* into subgenera, *Leishmania* and *Viannia*, was originally based on the vector gut parts colonized by the parasites (Lainson et al., 1977). Indeed, later on, this division was supported by DNA-sequence-based phylogenetic analyses (Croan et al., 1997; Noyes et al., 2002). *Leishmania* (*Leishmania*) parasites are transmitted to either female *Phlebotomus* (Old World) or *Lutzomya* (New World) species while *Leishmania* (*Viannia*) species are only found in the New World and therefore all the vectors are *Lutzomya* species (Bates, 2007). The ingestion of a blood meal containing *Leishmania* amastigotes by the female sandfly induces the amastigotes' transformation into procyclic promastigotes, a weakly motile and replicative form that multiplies in the blood meal confined by the peritrophic matrix, a mesh of proteins and chitin secreted by the insect midgut. Few days later, the parasites slow their replication and differentiate into strongly motile promastigotes that break the blood meal's peritrophic matrix and migrate. This escape is facilitated by the action of a

parasite secreted chitinase (Schlein et al., 1991; Shakarian and Dwyer, 2000). The species belonging to the subgenus *Leishmania* move towards the anterior midgut and some attach to the microvilli of the midgut's epithelium, while the species belonging to the *Viania* subgenus can be found in the pyloric region of the hindgut (Walters et al., 1989; Nieves and Pimenta, 2000). Differences in the ability to inhibit or to resist killing by proteolytic enzymes released into the vector midgut after blood feeding, and/or to remain in the gut during excretion of the digested blood meal define vector competence in most species. Indeed, the binding is mediated by the promastigotes' surface LPG that, displaying interspecies polymorphisms in their phosphoglycan domains, play a major role in specie-specific vector competence (Mahoney et al., 1999; Sacks et al., 2000; Kamhawi et al., 2000; Sacks et al., 2001).

The natural transmission of leishmaniasis is mediated by the bite of an infected female sandfly when she has a blood feeding. Indeed, at least as far as it is known, the infective inoculum includes, besides metacyclic promastigotes, sandfly saliva and the promastigotes' secretory gel (PSG). The PSG main component is a high molecular weight glycoprotein called filamentous proteophosphoglycan (fPPG) (Ilg et al., 1996). It plays a role in the parasite transmission and disease exacerbation, leading to increased pathology and parasite numbers when metacyclic promastigotes are co-inoculated with PSG (Rogers et al., 2002; Rogers et al., 2004). The sandfly saliva is required for the blood feeding due to its potent vasodilator and anti-haemostatic properties (Ribeiro, 1987). Furthermore, it is a disease exacerbation factor, at least for cutaneous leishmaniasis (Titus and Ribeiro, 1988). Co-inoculation of sandfly saliva and parasites led to disease exacerbation in several studies, which seems to be related with the modulation of the immune system to favor parasite survival and replication (Kamhawi, 2000; Rohousova and Volf, 2006). Indeed, salivary gland extracts inhibit macrophage activation, namely, the generation of nitric oxide (NO) as other oxygen-derived species and the antigen presentation to parasite reactive T cells (Titus and Ribeiro, 1990; Theodos and Titus, 1993; Hall and Titus, 1995). By contrast, the bites of uninfected sand flies or the vaccination with salivary gland extracts induce T cell mediated protection against an experimental challenge and disclose its potential for vaccination (Belkaid et al., 1998). Furthermore, in infected humans, anti-saliva antibodies are detected (Gomes et al., 2002).

Occasionally, the transmission of leishmaniasis does not involve sandflies. Visceral leishmaniasis can be induced through blood containing amastigotes (shared needles, transfusion, transplacental spread) or organ transplantation (Morillas-Marquez et al., 2002; Cruz et al., 2002; Pagliano et al., 2005).

1.4.2 Reservoirs and transmission modes

Leishmaniasis can be classified, depending on whether humans are the accidental or the direct hosts of the vector, as a zoonose or as an anthroponose. The several *Leishmania* species that cause disease in humans have a zoonotic transmission, with the exception of *L. donovani* and *L. tropica*, which have an anthroponotic transmission. However, even for these species, some animal reservoirs in endemic areas have recently been identified (Dereure et al., 2003; Jacobson et al., 2003; Mohebali et al., 2005).

Wild canines and domestic dogs were identified as the main reservoirs of zoonotic VL, while rodents and marsupials have been identified as the main reservoirs of CL. Furthermore, the different *Leishmania* species do not seem to exhibit reservoir strain or specie specificity, unlike what happens with the sandfly. Indeed, certain *Leishmania* species are maintained by several hosts, and several *Leishmania* species may be found in the same host (Saliba et al., 1988; Dereure et al., 1991; Strelkova et al., 1993; Ashford, 1996).

The most widespread zoonotic leishmaniasis is the VL caused by the single specie *L. infantum*. This disease is prevalent in the Central and South America, Mediterranean basin and Asia. Dogs are the main domestic reservoirs while jackals, foxes and wolves are the sylvatic ones (for review see Gramiccia and Gradoni, 2005). However, unusual *Leishmania* animal hosts such as cats and equines have recently been identified in those endemic areas (for review see Mancianti, 2004). The role of cats is still under debate, but they seem to be secondary reservoirs, while equines are incidental hosts (Gramiccia and Gradoni, 2005).

2 Leishmaniasis

2.1 Disease burden and geographical distribution

Leishmaniasis is a complex of diseases with an important clinical and epidemiologic diversity. Globally, leishmaniasis affects 2 million new cases and causes 70000 deaths each year, and 350 million people are at risk of infection and disease (WHO, 2004). Morbidity and mortality because of leishmaniasis cause an estimated 2.4 million disability-adjusted life-years (WHO, 2004). The large spectrum of clinical manifestations reflects leishmaniasis' complexity, mainly associated with the number of *Leishmania* species that cause disease, as well as many sandfly and mammalian species indicated as vectors and reservoirs, respectively.

2.1.1 Cutaneous leishmaniasis

Despite being endemic in more than 70 countries worldwide, 90% of cutaneous leishmaniasis cases occur in Afghanistan, Algeria, Brazil, Pakistan, Peru, Saudi, Arabia and Syria (Figure 5) (Desjeux P, 2000). However, surveillance data from Afghanistan (Reithinger et al., 2003), Bolivia (Davies et al., 2000), Brazil (Brandão Filho et al., 1999), Colombia (Davies et al., 2000; King et al., 2004), Peru (Davis et al., 2000) and Syria (Tayeh et al., 1997) indicate that the global number of cases has increased over the past decade. This could indeed be due, in part, to improved diagnostic and case notification, but inadequate vector and reservoir control, increased detection of cutaneous leishmaniasis associated with opportunistic infections (e.g. HIV) (Molina et al., 2003) and the emergence of drug-resistance (Croft et al., 2006) also contribute to such an increase.

Cutaneous leishmaniasis in endemic areas affects mainly children of up to 15 years of age, and the main risk factors besides age are household design and construction materials, and the presence of domestic animals (Reithinger et al., 2003; Davies et al., 2000; Desjeux, 2001).

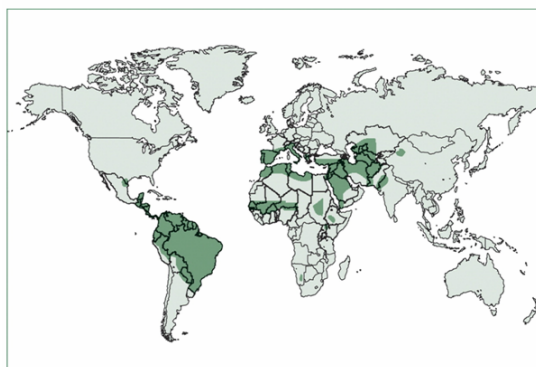


Figure 5. Worldwide distribution of the cutaneous leishmaniasis. 90% of CL occurs in Afghanistan, Algeria, Brazil, Pakistan, Peru, Saudi, Arabia and Syria. (Adapted from Reithinger et al., 2007).

2.1.2 Mucocutaneous leishmaniasis

This form of the disease is characterized by the ability of the parasite to metastasise to mucous tissues by lymphatic or haematogenous dissemination. It is usually caused by *L. braziliensis* and thus represents a considerable healthcare problem in Latin America (Davies et al., 2000). However, other *Leishmania* species such as *L. amazonensis* (Barral et al., 1991), *L. major* (Kharfi et al., 2003), *L. tropica* (Morsy et al., 1995) and *L. infantum* (Aliaga et al., 2003) have also been identified as etiological agents. In most endemic areas, 1-10% of localized CL infections led to MCL, 1-5 years after healed (Davies et al., 2000). It is therefore recommended that CL is promptly treated to prevent mucosal metastasis.

2.1.3 Visceral leishmaniasis

VL, the systemic form of leishmaniasis, is caused by the *Leishmania* species belonging to the *Leishmania donovani* complex, namely *L. chagasi* (New World) *L. donovani* and *L. infantum* (Wold World) (Table I). There is growing evidence to suggest that *L. chagasi* and *L. infantum* are the same specie (Momen et al., 1987; Rioux et al., 1990; Cupolillo et al., 1994; Mauricio et al., 1999). Indeed, several authors refer to them as synonyms, despite the disagreement of others (Shaw, 1994; Lukes et al., 2007; Chappuis et al., 2007). The transmission of *L. infantum* and/or *L. chagasi* is zoonotic, while it is anthroponotic to *L. donovani* (Table I). While *L. donovani* infects all age groups, *L. infantum* infects mainly children and immunosuppressed individuals. More than 90% of VL cases occur in Bangladesh, India, Nepal, Sudan, Ethiopia and Brazil (Figure 6) (Chappuis et al., 2007). Indeed, the estimated number of VL new cases and deaths each year is, respectively, 500000 and 50000 (Desjeux et al., 2004). The increased incidence of the disease has been associated to migration, lack of control measures, and HIV co-infection

(Boelaert et al., 2000; Desjeux, 2001). Cases of HIV/*Leishmania* co-infection have been reported in 35 countries worldwide. The HIV/*Leishmania* co-infected individuals present a high risk of developing VL upon *Leishmania* infection due to their immunosuppressed status (Moreno et al., 2000). Furthermore, they present high parasite loads in organs, low sensitivity to the serological-based diagnostic tests and treatment failure (Murray et al., 1999; Deniau et al., 2003). Several cases of HIV/*Leishmania* co-infection have been reported in South-Western Europe (Desjeux, 2000).

PKDL, a VL complication that arises after treatment, is very frequent in Sudan and more rarely, in East African countries and Indian subcontinents (Zijlstra et al., 2003). PKDL cases are highly infective, since the nodular lesions contain many parasites, and they might represent a putative reservoir for anthroponotic VL transmission (Addy and Nandy, 1992).



Figure 6. Worldwide distribution of the visceral leishmaniasis. 90% of VL cases occur in Bangladesh, India, Nepal, Sudan, Ethiopia and Brazil (Adapted from Chappuis et al., 2007).

2.2 Diagnosis

The large clinical spectrum of leishmaniasis makes it difficult to diagnose. Furthermore, other pathologies with similar clinical manifestations (e.g. leprosy, skin cancers, tuberculosis, cutaneous mycoses for CL and malaria or schistosomiasis for VL) are common in *Leishmania* endemic areas (Escobar et al., 1992). Therefore, specific and sensitive tests are very important, not only for a prompt and accurate diagnosis but also for a successful treatment and subsequent disease control.

2.2.1 Microscopy

The visualization of the parasite by microscopy examination of Giemsa-stained: lesion biopsy smears (for CL) or lymph nodes, spleen or bone marrow aspirates (for VL) remains the gold standard confirmatory diagnostic method for leishmaniasis in mainly endemic areas. Occasionally, the culture of biopsy triturates or aspirates is

also performed (Escobar et al., 1992). Even though it is very specific, the sensitivity of this method is highly variable and depends largely on the number and dispersion of parasites in the biopsy, the technical skills of the personnel, the sampling procedure and the sample origin (Herwalt, 1999). Indeed, in the diagnosis of VL, the sensitivity of the microscopy is higher for the spleen (93-99%), when compared to bone marrow (53-86%) or lymph node (53-65%) aspirates (Siddig et al., 1988). However, spleen aspirates require considerable technical expertise, since life threatening haemorrhages can occur (Kager et al., 1983).

2.2.2 PCR

Among the molecular biology methods available to detect *Leishmania* parasites, the most used are the PCR-based ones. The PCR revealed to be very specific and sensitive in the detection of *Leishmania* parasites. It has been considered a better approach in samples with low parasite load (MCL) (Garcia et al., 2005) and from less invasive methods such as blood (Cruz et al., 2002) as well as conjunctive (Strauss-Ayali et al., 2004) than the conventional microscopy. The relevance of this technique is extended to the diagnosis of *Leishmania* in HIV co-infected individuals (Cruz et al., 2002; De Doncker et al., 2005). Furthermore, the possibility of identifying the *Leishmania* specie and predicting the disease severity and treatment outcome is becoming important in the patients' clinical management (Murray et al., 2005). Genetic markers of high copy number (e.g. rRNA genes, kinetoplast DNA minicircles, mini-exon genes) (Bensoussan et al., 2006) are usually selected to perform detection, quantification and viability studies, while repeated and polymorphic sequences are targeted to perform species identification (e.g. gp63, hsp70, and cysteine proteinases) (Garcia et al., 2005).

However it must be stressed that the feasibility of PCR diagnosis in endemic countries is still limited, since it requires high equipment and working costs.

2.2.3 Serological tests

Several tests for the detection of anti-*Leishmania* specific antibodies have been developed. Their applicability in the diagnosis of CL, however, is rare due to the variable specificity and sensitivity (Kar, 1995), and in the diagnosis of VL there are two main limitations. One is related to the persistence of the anti-*Leishmania* antibodies for long periods of time after the cure (Hailu, 1990; De Almeida Silva et al., 2006) and the other has to do with the detection of anti-*Leishmania* antibodies in healthy individuals from endemic areas with no story of VL (Sundar et al., 2006). These tests should therefore be used in combination with clinical symptoms for an

accurate diagnosis of VL. The several limitations of the poor *Leishmania* endemic areas have boosted the development of diagnostic tests that could be used in the field: easy to perform, cheap, reproducible and rapid. Therefore, two serological tests have been adapted and validated to field conditions – the fast agglutination screening test (FAST), a modified version of the direct agglutination test (DAT), and the rK39-immunocromatography or dipstick based test (ICT) (Schoone et al., 2001; Silva et al., 2005; Hailu et al., 2006; Chappuis et al., 2007). While the agglutination tests are based on the use of *L. donovani* stained promastigotes that agglutinate in the presence of a serum containing anti-*Leishmania* antibodies (el Harith et al., 1988), the rK-39 dipstick uses a recombinant 39-amino acid repeat that is part of a kinesin-related protein in *L. chagasi* and which is conserved within the *L. donovani* complex (Burns et al., 1993). The diagnostic performance of the latter has been evaluated in the several VL endemic areas (Chappuis et al., 2006; Sundar et al., 2007; Ritmeijer et al., 2007; Boelaert et al., 2008).

2.2.4 Antigen detection

Among the limitations of the antibody detection diagnostic-based tests is the impossibility to distinguish between an acute and asymptomatic disease and the antibodies' cross-reactivity. An antigen-based diagnostic test has been developed for VL. Indeed, it is based on the detection of a heat-stable, low-molecular-weight carbohydrate antigen in the urine of VL patients (Attar et al., 2001; Sarkari et al., 2002). Although, improvements concerning the test sensitivity are required (Chappuis et al., 2006; Sundar et al., 2007).

2.3 Disease control

In general, the control of leishmaniasis is based on case detection followed by treatment, but also on vector and reservoir control, since no effective anti-*Leishmania* vaccine is available. A brief overview of each approach will be given, but treatment options, due to their relevance in the scope of this thesis, will be dealt with in more detail in the next chapter.

2.3.1 Control of vectors and reservoirs

Since sandflies are susceptible to insecticides, spraying houses with them is the most widely used intervention to control sandflies that are endophagic (mainly stay indoors after feeding) and endophilic (mainly feed indoors). The house spraying

campaigns carried out with dichloro-diphenyl-trichloroethane (DDT) during the 1950s to eradicate malaria almost led to the disappearance of VL in India. However, once the campaigns were discontinued, the disease re-emerged. Due to the hazardous effects of DDT on humans and the environment, it was substituted by synthetic pyrethroids, like deltamethrin and cyhalothrin (Marcondes and Nascimento, 1993). Indeed, house spraying with pyrethroid lambdacyhalothrin reduced CL in Kabul by 60% (Reyburn et al., 2000) and the risk of CL in the Peruvian Andes by 54% (Davies et al., 2000). The use of insecticide-impregnated materials, such as bednets, curtains, clothes or bed-sheets, has also proved effective in the control of CL (Reyburn et al., 2000; Kroeger et al., 2002). However, there are some endemic regions, such as Sudan and East Africa, where this type of control measures is not effective, since the vector feeding occurs mainly outside (Hassan et al., 2004). Furthermore, economic constraints limit the efficiency and applicability of these control measurements.

In the regions where dogs are domestic reservoirs of *Leishmania*, the use of deltamethrin impregnated collars revealed to be effective in reducing the risk of infection in dogs and children (Reithinger et al., 2004 and 2006). This strategy seems feasible and effective, since the killing of sero-positive animals is not well accepted by ethical reasons and its efficiency is even debated (Ashford et al., 1998; Palatnik-de-Sousa et al., 2001; Reithinger and Davies, 2002). Furthermore, the treatment of infected dogs is not an efficient measure not only due to frequent relapses but also to the development of drug resistances (Alvar et al., 1994; Gavgani et al., 2002).

2.3.2 Vaccination

The idea that it will be possible to generate a vaccine against leishmaniasis is provided by the evidence that most individuals that had leishmaniasis or a symptomless infection are resistant to subsequent clinical infections. The need for a safe and effective vaccine is made greater due to the treatment's toxicity, the emergence of drug resistances and the increased incidence of the disease in immunocompromised individuals. Indeed, substantial efforts have been made to develop human vaccines for the prevention of CL and VL, and dog vaccines, to control the zoonotic transmission of VL.

The inoculation of uninfected individuals with live parasites, leishmanization, was the first vaccination approach, and it was carried out in Israel, Russia and Uzbekistan. However, its use is at present limited to one vaccine registered in Uzbekistan, and to live challenge in vaccine efficiency trials to humans in Iran

(Khamesipour et al., 2006). Several reasons, such as the spread of HIV, the use of immunosuppressive drugs, the uncontrolled long-lasting skin lesions and the parasite persistence, have led to the discontinuity of leishmanization. Progressively, the so-called “killed vaccines”, composed mainly by crude parasite antigens, have reached the human clinical trials and were classified as first-generation vaccines. Indeed, most of the trials were developed against CL (Momeni et al., 1999; Armijos et al., 2004; Velez et al., 2005), while only one was directed to VL (Khalil et al., 2000). However, the average efficacy value of these vaccines was low (Palatnik-de-Sousa, 2008). This first-generation of vaccines was also tested against canine visceral leishmaniasis, leading to protection in Iran (Mohebbi et al., 2004) but failing to do so in Brazil (Genaro et al., 1996). These vaccines have been successful for immunotherapy in combination with classical chemotherapy in Brazil (Mayrink et al., 2006) and Venezuela (Convit et al., 1987). Indeed, a first-generation vaccine was registered in Brazil as adjunct to antimony therapy (Mayrink et al., 2006). The second-generation vaccines have been based on the following main types: (i) live vaccines; (ii) purified *Leishmania* antigens and (iii) recombinant antigens.

(i) Live vaccines include: genetically modified *Leishmania* parasites containing suicidal cassettes such as the *L. major* expressing the ganciclovir-sensitive thymidine kinase gene of Herpes virus (Muyombwe et al., 1998); recombinant bacteria or virus expressing *Leishmania* antigens such as the *Vaccinia* virus expressing the *L. infantum* LACK antigen (analogue parasite to the receptor of activated mammalian kinase C) which protects mice against *L. major* (Gonzalo et al., 2002) and dogs against *L. infantum* (Ramiro et al., 2003); and genetically modified mutant *Leishmania* parasites carrying deletions on genes that led to an attenuated virulence and protection against a virulent challenge, such as the deletion of the gene encoding to dihydrofolate reductase-thymidylate synthase (DHFR-TS) in *L. major* (Cruz et al., 1991; Titus et al., 1995), cysteine protease in *L. mexicana* (Souza et al., 1994; Mottram et al., 1996; Alexander et al., 1998), bipterine transporter in *L. donovani* (Papadopolou et al., 2002), LPG2 in *L. major* (the gene encoding Golgi GDP-Mannose transporter) (Spath et al., 2003; Uzzona et al., 2004) and SIR2 in *L. infantum* (the gene encoding a silent information regulator 2 homologue protein) (Silvestre et al., 2007).

(ii) Another approach to develop second-generation vaccines includes the purification of *Leishmania* sub-fractions. Indeed, a dog vaccine containing a glycoprotein-enriched preparation of *L. donovani* promastigotes, named Fucose-Mannose-ligand (FML), formulated with saponin became a licensed vaccine in Brazil

called Leishmune® (Parra et al., 2007; Santos et al., 2002; Palatnick de Sousa et al., 1994; Santos et al., 2003).

(iii) Over the last few years, several *Leishmania* recombinant proteins alone, in combination or as polyproteins or chimeras, have been intensively tested. None of them have reached Phase III dog's clinical trials (Palatnik-de-Sousa, 2008). Indeed, most of these vaccine candidates were tested in mice model and only a few advanced to monkey trials for CL (Masina et al., 2003; Campos-Neto et al., 2001) or to kennel assays in dog models of VL (Fujiwara et al., 2005; Gradoni et al., 2005; Moreno et al., 2007; Poot et al., 2006; Molano et al., 2006) or even to pre-clinical human assays (Badaro et al., 2006).

The third-generation vaccines are composed of DNA vaccines. Their main advantages, when compared to recombinant proteins, are related to their stability, low production cost, and the flexibility of combining multiple genes in one construction. The most studied antigens are those previously assessed as recombinant proteins (Palatnik-de-Sousa, 2008). Most of them were tested as single vaccines (Ghosh et al., 2001; Fragaki et al., 2001; Melby et al., 2001; Sukumaran et al., 2003), and some as a combination of genes (Campos-Neto et al., 2002; Iborra et al., 2004; Rafati et al., 2001), or as an heterologous prime-boost involving the DNA vaccine followed by the injection of the recombinant protein (Rafati et al., 2005). Despite the protection achieved with the DNA vaccines, no data is available from Clinical Phase III trials. Some of the most promising candidates, however, are the *Leishmania* homologue of receptors for activated C kinase (LACK), *Leishmania* elongation initiation factor (LeIF), thiol-specific antioxidant (TSA), *L. major* stress-inducible protein 1 (LmSTI1) and histone H1 (H1), (Dumonteil, 2007; Palatnik-de-Sousa et al., 2008).

Chapter II

Host-parasite interactions

The *Leishmania* parasite is a medically important pathogen that targets macrophages for the establishment of persistent infections. Macrophages play a central role in the host's response to infections, promoting the destruction of pathogens and priming the host's immune response. However, *Leishmania* has adopted strategies to exploit macrophage functions into promoting its own survival.

1 Macrophages

1.1 Phagocytosis

Leishmania metacyclic promastigotes and sandfly saliva are delivered in the mammalian host's upper dermis during the blood-feed of an infected sandfly (Schelein et al., 1992). Immediately after entering the blood, and before being engulfed by a phagocytic cell, promastigotes have to evade the complement mediated cell-lysis. The procyclic *Leishmania* promastigotes (non infective) are extremely sensitive to complement action, even in the absence of antibodies, while infectious metacyclic promastigotes can fully avoid complement mediated lysis. This is explained by the action of surface LPG, which is significantly longer in the metacyclics when compared to the non-infective forms, and will prevent the attachment of C5b-C9 complement subunits to the parasite surface (Puentes et al., 1990; McConville et al., 1992). In addition, protein kinases at the cell surface were shown to phosphorylate several components of the complement system thus inhibiting the cascade (Hermoso et al., 1991). While around 10-fold less abundant than LPG, the surface glycoprotein, gp63, is also involved in the resistance to complement mediated lysis. This parasite protease cleaves the complement component C3b into iC3b that, deposited on the parasite's surface, are recognized by the macrophages' complement receptors 1 (CR1) and CR3 respectively, and thus facilitate the parasite's internalization (Mosser and Edelson, 1987; Brittingham et al., 1995). However, the most important macrophage complement receptor is the CR3, since the binding to CR1 is transient due to the immediately conversion of C3b in iC3b by gp63 (Kane and Mosser, 2000). Furthermore, attachment through CR3 does not activate the oxidative burst during phagocytosis (Mosser and Edelson, 1987). Moreover promastigotes can also interact by LPG with the macrophage mannose-fucose receptors (Blackwell et al., 1985); LPG can be also recognized by the C-reactive protein macrophage receptor (CRP) (Culley et al., 1996) or by the

CR4 macrophage receptor (Talamas-Rohana et al., 1990). Furthermore gp63 is able of interact with the fibronectin macrophage receptor (Brittingham et al., 1999).

The *Leishmania* parasites which are internalized by macrophages through phagocytosis are trafficked to endosomes and lysosomes. The resulting phagolysosome, also called parasitophorous vacuole (PV), is an acidified and hydrolytically active compartment where the *Leishmania* parasite is not only capable to survive but also to multiply (Chang et al., 1976; Antoine et al., 1998). The parasite's persistence in such a hostile environment is attributed to its differentiation in amastigotes, which resist to macrophage hydrolases (Desjardins et al., 1997). Thus, while acid-resistant forms are being generated, several mechanisms have been proposed to explain the role of LPG retarding phagosome maturation in *L. major* and *L. donovani* (Old World) species (reviewed by Lodge and Descoteaux, 2005). Indeed, LPG-repeating units interfere with F-actin's normal turnover, preventing the interaction with late endosomes and lysosomes (Holm et al., 2001). Furthermore, LPG seems to prevent the recruitment of lipid rafts, which are enriched in flotinin, or cholera toxin B subunit ganglioside GM1 and proteins involved in membrane fusion, such as NADPH oxidase, as well as PKC isoenzymes, to the phagosome membrane (Lang et al., 2002; Vilhardt et al., 2004; Dermine et al., 2005). By contrast, parasites from the *L. mexicana* complex (New World) do not affect phagosome biogenesis and reside within enormous PV that harbour many amastigotes, when compared to phagosome membranes harbouring single amastigotes of Old World species (Ilg et al., 2000; Duclos et al., 2000). It has been suggested that the large vacuole size might dilute the hydrolytic enzymes to a level below their effectiveness, therefore being an alternative intrinsic mechanism favouring the promastigote differentiation (Duclos et al., 2000). Recently, a calveolae-mediated mechanism for the internalization of *L. chagasi* promastigotes was described (Rodriguez et al., 2006). This mechanism, involving cholesterol containing macrophage membrane microdomains, targeted *L. chagasi* promastigotes to a compartment showing delayed interactions with lysosomes, thus promoting parasite survival.

It has been suggested that an indirect way of silently delivering promastigotes to macrophages would include the parasite's uptake by neutrophils (Laskay et al., 2003). Neutrophils are primary antimicrobial effector cells of the innate immune system, capable of destroying invading pathogens. These cells participate in the early clearance of pathogens by phagocytosis since immediately after the phagosome fuses with cytosolic granules containing hydrolytic enzymes and bactericidal proteins. In addition, these cells can generate an oxidative burst to destroy engulfed pathogens (Segal, 2005). Furthermore, a novel neutrophil-

mediated antibacterial mechanism includes the release of neutrophil extracellular traps (NETs). These extracellular structures, produced by activated neutrophils, containing DNA, histones and antibacterial enzymes, are effective in the killing of bacteria and fungi (Brinkmann and Zychlinsky, 2007). However, some pathogens ingested by neutrophils can survive within. Indeed, *Leishmania* promastigotes are silently ingested by neutrophils, since no activation of the oxidative burst occurs (Laufs et al., 2002). This silent entry has been associated to the presence of some apoptotic parasites in the infectious inoculum that assists the survival of non-apoptotic parasites (van Zandbergen et al., 2006). Furthermore, *Leishmania* infected neutrophils display an extended life span through apoptosis inhibition (van Zandbergen et al., 2004). When infected neutrophils exhibit the phosphatidylserine (PS) signal, they are readily phagocytosed by macrophages and the *Leishmania* parasites within them not only survive but are also able to multiply (van Zandbergen et al., 2004). Based on this, neutrophils are a perfect temporary shelter to preserve *Leishmania* parasites from a hostile environment before they enter macrophages. In the case of *Leishmania* amastigotes, it is well accepted that they enter phagocytic cells mainly through Fc and complement receptors (Guy and Belosevic, 1993; Peters et al., 1995; Kima et al., 2000). Moreover, amastigotes can also gain silent access into macrophages through PS externalization (Fadok et al., 2000). The recognition of PS on the membrane of apoptotic cells suppresses phagocyte activation in a TGF- β dependent manner (Voll et al., 1997). However, the mechanism of how amastigotes infect non-phagocytic cells is not well understood.

1.2 Modulation of macrophage function

The presence of *Leishmania* parasites in macrophages allows their evasion from the host's immune system. However, it is necessary to inhibit several macrophage functions, mainly involved in immune surveillance and macrophage activation. The repressed functions can be grouped into three main types:

- (i) Microbicidal functions;
- (ii) Cytokine production;
- (iii) Antigen presentation and effector's cell activation;

(i) Microbicidal functions

Macrophages can produce two distinct types of molecules which are effective against *Leishmania*, nitric oxide (NO) (Liew et al., 1990) and reactive oxygen species (ROS) (Murray, 1982). NO is produced by the inducible Nitric Oxide Synthase (iNOS) and seems to play an important role in the clearance of *Leishmania*. Indeed, a *Leishmania*-resistant strain of mouse, when lacking the gene that encodes iNOS, became susceptible to *L. major* infection (Wei et al., 1995; Murray and Nathan, 1999). By contrast, mice deficient in the generation of ROS can control the infection, after an initial period of increased susceptibility (Murray et al., 1999). However, *Leishmania* can inhibit the production of ROS by macrophages (Buchmuller-Rouiller and Mael, 1986; Olivier et al., 1992). This has been related with the inhibition of protein kinase C (PKC) activation, through the interaction of the parasite surface molecules, LPG and gp63, with macrophages (Olivier et al., 1992; Sorensen et al., 1994; Descoteaux and Turco, 1999).

(ii) Cytokines production

The production of cytokines, mainly pro-inflammatory, such as IL-1, TNF- α and IL-12, is repressed in *Leishmania* infected macrophages. The secretion of IL-1 induced by LPS is inhibited in macrophages infected with *L. donovani* or *L. major*, or alternatively treated with LPG (Reiner, 1987; Frankenburg et al. 1990). Interestingly, the IL-1 β production is repressed at the level of gene expression while repression of IL-1 α is post-transcriptionally regulated (Hatzigeorgiou et al. 1996; Hawn et al. 2002). The TNF- α expression is also inhibited in macrophages infected with *L. donovani* and seems to be related with the secretion of the immunosuppressive cytokine IL-10 (Bhattacharyya et al. 2001). The mechanism involved in the repression of IL-12 is not fully understood, even though it occurs after the engagement of complement and Fc macrophage receptors (Marth and Kelsall, 1997; Sutterwala et al., 1997). The repression of this cytokine production has been demonstrated *in vitro* for *L. donovani* and *L. major* promastigotes (Carrera et al., 1996), *L. mexicana* amastigotes (Weinheber et al., 1998) and the phosphoglycan fraction of LPG (Piedrafita et al., 1999). This was also demonstrated in *L. major* infected mice (Belkaid et al., 1998). Indeed, IL-12 is an important cytokine involved in lymphocyte activation with subsequent production of INF- γ leading to macrophage activation and production of microbicidal molecules.

Furthermore, *Leishmania* parasites can induce the production of suppressive signalling molecules, such as arachidonic acid metabolites, IL-10 and TGF- β cytokines.

TGF- β has been described as a cytokine produced by macrophages infected with several *Leishmania* species, both *in vitro* and *in vivo*. This cytokine will suppress the expression of iNOS by macrophages in an autocrine and paracrine manner (Stenger et al., 1994) and inhibit the production of INF- γ by NK cells (Kaye and Bancroft, 1992; Wilson et al., 1998). Indeed, a study of *L. chagasi* infection has demonstrated that human-infected macrophages secrete active TGF- β , which is produced from the cleavage of pro-TGF- β by the amastigote's cysteine proteases (Gantt et al., 2003; Somanna et al., 2002).

IL-10 is considered to be the most immunosuppressive cytokine in visceral leishmaniasis and a powerful macrophage deactivator, as it suppresses NO production and the secretion of pro-inflammatory cytokines (IL-1, TNF- α and IL-12) (Cunningham, 2002). The secretion of both cytokines by infected macrophages is also induced by the uptake of amastigotes exhibiting PS at their surface. A similar immunosuppressive role was proposed for the arachidonic acid metabolite, PGE₂, which is generated by *Leishmania*-infected macrophages, both *in vitro* and *in vivo* (Reiner and Malemud, 1985; Matte et al., 2001).

(iii) Antigen presentation and effector's cell activation

The development of an effective immune response against *Leishmania* requires an efficient antigen presentation to T lymphocytes. Even though dendritic cells are recognized as the most important antigen-presenting cells (APC), macrophages also present antigens through major histocompatibility complex (MHC) class I and II. Although a protective role against *Leishmania* infection has been shown for MHC class I antigen presentation, through the induction of effector CD8⁺ T cells (Uzonna et al., 2004), only the presence of MHC class II presenting cells is essential for complete parasite clearance (Locksley et al., 1993). Indeed, several studies have revealed that *Leishmania* inhibits macrophage MHC II antigen presentation and different mechanisms were being proposed (reviewed in Gregory and Olivier, 2005).

An effective T cell activation requires, besides the signal delivered by the peptide antigen, a second signal provided by surface molecules on the APC, known as co-stimulatory molecules, such as B7/CD28 and CD40/CD40L. After *Leishmania* infection, the macrophages cannot express B7-1 (CD80) upon LPS stimulation in a prostaglandin-dependent process (Saha et al., 1995; Pinelli et al., 1999). Furthermore, B7-CD28 interconnection has been proved important in mice resistance against leishmaniasis (Corry et al., 1994). In addition, mice deficient on CD40 or CD40L or with CD40-CD40L inhibited, exhibit a more susceptible

phenotype towards a *Leishmania* infection, which was correlated with diminished secretions of IFN- γ and IL-12 (Campbell et al., 1996).

1.3 Cell signalling

As described in the previous section, *Leishmania* can modulate the macrophage function due to an interference with the cell signalling pathways. These are commonly triggered by the ligation of an external ligand (e.g. *Leishmania* molecule; cytokine) to a receptor on the cell surface that became activated, commonly by phosphorylation and/or conformational changes, and leads to the activation of second messengers within the cytosol. The second messengers are often kinases, which then phosphorylate other kinases to continue a cascade that ultimately results in the activation of effector molecules, such as transcription factors or actin filaments, and causing a change in the cell's behaviour. Some examples of the macrophage-affected signalling pathways by *Leishmania* infection will be given and include: Ca²⁺ and PKC dependent pathways; mitogen-activated protein (MAP) kinases pathway; JAK/STAT and INF- γ mediated signalling and Protein Tyrosine Phosphatase, SHP-1.

PKC and Ca²⁺ have been described as second messengers modulated by a *Leishmania* infection. The Ca²⁺ intracellular concentration was shown to be augmented upon *Leishmania* infection (Eilam et al., 1985). Both *Leishmania* and LPG were described to have an active role in increasing the intracellular Ca²⁺ of phagocytes, altering the Ca²⁺ dependent responses as chemotaxis and ROS production (Eilam et al., 1985; Olivier et al., 1992). The PKC activity has been shown to be reduced in *Leishmania*-infected macrophages favouring the parasite's survival (McNeely and Turco, 1987).

Several important macrophage functions against pathogens (e.g. NO production, MHC II antigen presentation) are activated by INF- γ through the JAK2/STAT1 signalling pathway. Indeed, it is not surprising that *Leishmania*-infected macrophages have shown an inhibition of this pathway upon INF- γ stimulation (Nandan and Reiner, 1995; Blanchette et al. 1999).

The infection of naive macrophages by *Leishmania* represses the most important mitogen-activated protein kinase (MAPK) family members such as: Extracellular-Regulated Kinase (ERK1/2), p38 and Jun N-terminal Kinase (JNK), which is consistent with a parasite silent entry (Prive and Descoteaux, 2000). The MAP kinases play an important role in the immune response to *Leishmania*. The inhibition of ERK1/2 and p38 MAPK that occurs in macrophages upon *Leishmania* infection, due to the activation of a host protein tyrosine phosphatase (PTPase)

called SHP-1, leads to the suppression of pro-inflammatory cytokines and NO, favoring the parasite's survival (Olivier et al., 2005). Moreover, the infection of mice deficient in SHP-1 has disclosed its role in the progression of leishmaniasis, since these animals were resistant to *Leishmania* infection and it was correlated with an efficient NO production (Matte et al., 2000; Forget et al., 2001).

2 Dendritic cells

Dendritic cells (DC) have a central role in the induction of immunity and peripheral tolerance (Steinman et al., 2000; Lutz and Schuler, 2002). However, the mechanisms of how DCs initiate or redirect an effective immune response to eliminate pathogens or tumour cells and induce self tolerance are not well known. Of particular interest is the ability of DCs to induce naïve Th cell differentiation into different Th cell responses depending on the maturation stimuli (de Jong et al., 2002; Manickasingham et al., 2003). Thus the type of DC stimulation may determine the DC-mediated polarization of Th cell responses. DCs sense inflammatory and microbial signals leading to their maturation, as expressed by the up-regulation of MHC and co-stimulatory molecules (Menges et al., 2002; Sporri and Reis e Sousa, 2005). The secretion of IL-12 by DCs seems to be the major factor inducing Th1 polarization (Moser and Murphy, 2000).

The infection of DCs by *Leishmania* has been implicated in the parasite's dissemination (Moll et al., 1993). Several studies using DCs from different sources, such as mouse bone marrow-derived DC, mouse skin-derived Langerhans cells or human monocyte-derived DCs, have been performed, to understand how DCs respond to *Leishmania* parasites and antigen specific CD4⁺ T cells are activated. The completion of *in vitro* studies revealed that bone marrow-derived DCs but not Langerhans cells can efficiently engulf *Leishmania* promastigotes, becoming activated as measured by the little secretion of IL-12 p70 but a range of IL-12 p40 and IL10 (von Stebut et al., 2000; Qi et al., 2001; Xin et al., 2007). Although, depending on the parasite specie, infection leads to a reduction of DC responsiveness to exogenous stimuli such as LPS and INF- γ (von Stebut et al., 2000; Xin et al., 2007). However, the infection of DC by *Leishmania* amastigotes does not induce DC maturation and seems to occur through C-type lectin, ICAM-3-grabbing nonintegrin receptor (DC-SIGN) at least for *L. infantum*, *L. pifanoi*, *L. donovani* and *L. amazonensis* but not *L. major* (Bennett et al., 2001; Brandonisio et al., 2004; Colmenares et al., 2002; Colmenares et al., 2004; Caparros et al., 2005). Moreover, antibody opsonization promotes the uptake of *L. major*

amastigotes by mouse bone marrow-derived DCs and skin-derived Langerhans cells (von Stebut et al., 1998; Woelbing et al., 2006). Indeed, antibody opsonization of amastigotes (or promastigotes) enhances DC activation and production of IL-10 (and, to some extent, IL-12 p40), leading to the preferential priming of IL-10 producing CD4⁺ T cells and consequent lesion progress in mice (Wanasen et al., 2008 ; Prina et al., 2004). In contrast, a protective immunity mediated by DCs secreting IL-12 has been described for the uptake of antibodies-opsonized *L. major* amastigotes by mouse bone marrow-derived DCs or lesion derived *L. donovani* amastigotes by human myeloid DCs (Woelbing et al., 2006; Ghosh et al., 2006). Several studies have been performed to disclose the initial interactions of *Leishmania* promastigotes and DCs *in vivo*. Even though plasmacytoid DCs can be detected in *Leishmania* infected skin and they may not internalize promastigotes, they can be activated by the released *Leishmania* genomic DNA to produce INF- α and IL-12 in a toll-like receptor 9 (TLR9)-dependent manner (Schleicher et al., 2007). However, myeloid DCs and IL-12, rather than plasmacytoid DCs and INF- $\alpha\beta$, are required for NK cell cytotoxicity and INF- γ production in visceral and cutaneous leishmaniasis (Bajenoff et al., 2006; Schleicher et al., 2007). Once myeloid DCs and monocyte DCs efficiently engulf promastigotes, they can serve as a source of IL-12. Activated DCs can migrate to draining lymph nodes carrying parasite antigens and activate NK-cells to produce INF- γ thus activating Th1 effector cells (Ritter et al., 2004; Ritter et al., 2007; Leon et al., 2007).

3 Immune response deviation

Experimental infections using *L. major* and inbred strains of mice revealed a correlation between the disease's phenotype and the cytokines' profile. Indeed, resistance to infection was associated with the expansion of a CD4⁺T cells subset expressing a Th1 phenotype, thus producing INF- γ and IL-2. On the other hand, susceptibility to infection was associated with the expansion of a CD4⁺T cells subset expressing a Th2 phenotype, thus producing IL-4 (reviewed in Peters and Sacks et al., 2006). Furthermore, the balance between Th1 and Th2 cytokines, produced in the context of *Leishmania* infection, seemed to dictate the infection outcome. Furthermore, the deviation of the host's immune response to a Th2 phenotype favors the parasite's persistence, not only because it occurs at the expenses of the host's protective Th1 phenotype but also because it promotes the parasite's growth, since Th2 cytokines induce arginase 1, that catalyses the hydrolysis of L-arginine

into urea and L-ornithine. The latter is used by the parasite to synthesize polyamines that are essential for its growth (Kropf et al., 2005).

However, subsequent studies suggested that Th2 phenotype polarization in *L. major*-infected Balb/c mice seemed to be due to multiple genetic defects that prevent them from redirecting the early IL-4 response to a Th1 differentiation during infection. Indeed, the early production of IL-4 during infection was confined to an oligoclonal population of CD4⁺ T cells with the V β 4 V α 8 TCR, that recognized the *Leishmania* antigen receptor for kinase C (LACK) (Launois et al., 1997; Malherbe et al., 2000). However, further studies revealed that the frequency of LACK MHC class II tetramer binding IL-4⁺ cells, induced early by *L. major* infection, was similar in susceptible and resistant mouse strains, and that infection with LACK-deficient *L. major* mutants (unable to activate LACK-specific V β 4 V α 8 CD4⁺ T cells) still induced Th2 development in infected mice (Stetson et al., 2002; Kelly and Locksley, 2004). Furthermore, the ability of exogenous IL-12 to redirect Balb/c early Th2 response to *L. major*, suggests that mice susceptibility was due to some intrinsic failure in this pathway (Sypek et al., 1993; Heinzel et al., 1993). Moreover, a number of intrinsic defects of this mice strain into developing a Th1 response have been described (Hondowicz et al., 2000; Mohrs et al., 2000; Filippi et al., 2003). Based on the several studies in mice, the control of parasites and development of acquired resistance are dependent on the production of IL-12 by the APCs and INF- γ by T cells (Engwerda et al., 1998; Bacellar et al., 2000; Caldas et al., 2005).

3.1 The role of IL-10

Mammalian host protection against leishmaniasis depends on the development of a Th1 immune response, since it triggers enhanced leishmanicidal activity by macrophages.

Human visceral leishmaniasis, the most clinically severe form of the disease, has been mainly associated with increased levels of IL-10 instead of IL-4 (Ghalib et al., 1993; Holaday et al., 1993). Moreover IL-10 and INF- γ have been implicated in the development of post-kala-azar dermal leishmaniasis (PKDL), and IL-4, IL-10 and INF- γ production detected in the more severe forms of cutaneous leishmaniasis (Ismail et al., 1999; Gasim et al., 2000). The role of IL-10 in the unfavorable progression of leishmaniasis has also been demonstrated for the mice model, since the deletion of the IL-10 locus or the treatment with anti-IL-10 antibody led to a better control of infection, even for the Balb/c mice infected with *L. major* (Kane et al., 2001; Noben-Trauth et al., 2003). Moreover, both Balb/c and C57BL/6 IL-10

deficient mice are highly resistant to *L. donovani* infection (Murphy et al., 2001; Murray et al., 2003). Furthermore, an *L. amazonensis* non-healing cutaneous lesion in C57BL/6 or C3H mice does not require IL-4, but it was significantly reduced in IL-10 deficient mice (Jones et al., 2002; Ji et al., 2003; Ji et al., 2005). These clinical and experimental data indicate that an unfavorable infection outcome is not related with an unbalanced Th1/Th2 phenotype but with the concomitant production of IL-10. The suppressive functions of this cytokine include the inactivation of macrophages and dendritic cells, with a consequent inhibition of pro-inflammatory cytokine secretion and the inhibition of MHC class II and co-stimulatory molecules expression (Moore et al., 2001). IL-10 inhibits the maturation of dendritic cells from monocyte precursors, and interferes with the ability of macrophages to kill intracellular pathogens, in part by inhibiting TNF production (Allavena et al., 1998; Moore et al., 2001). Therefore, the production of IL-10 by Th1 or Th2 cells has been suggested as an important feedback regulator controlling the pathology associated with an exaggerated, even effective, inflammatory response (O'Garra and Vieira P., 2007).

3.2 Sources of IL - 10

Originally, IL-10 was thought to be specifically produced by Th2 cells to inhibit Th1 responses. However, it became clear that this regulatory cytokine can be produced by many other cell types, including B cells, macrophages, dendritic cells, endothelial cells, mast cells, eosinophils and several T cell subsets, such as CD8⁺T cells, CD4⁺CD25⁺FOXP3⁺ cells (TReg cells, generated during thymic selection) and antigen-driven regulatory CD4⁺CD25⁺FOXP3⁺ cells (Tr1, emerged after an encounter with an antigen) (Moore et al., 2001; O'Garra and Vieira, 2004; Peters and Sacks et al., 2006). Indeed, the expansion and accumulation of IL-10- producing regulatory T cells have been associated with many chronic infections such as *Leishmania*, HIV and HCV. In the mice model of cutaneous leishmaniasis, TRegs modulate the development of effector responses and parasite persistence in the skin after clinical cure (Belkaid et al., 2002). However, in the case of human visceral leishmaniasis, the suppression of anti-*Leishmania* specific immunity seems to be controlled by Tr1 cells, since high levels of splenic IL-10 mRNA was found in the CD4⁺CD25⁺FOXP3⁺ cells (Roncarolo et al., 2001). Moreover, in *L. donovani*-infected mice, the production of IL-10 by splenic CD4⁺CD25⁺ T cells is strongly correlated with disease progression (Nylén et al., 2007).

It has been recently demonstrated that antigen specific INF- γ Th1-producing cells are also an important source of IL-10, which suppresses immunity into a non-

healing form of *L. major*, thus preventing lethal inflammation (Anderson et al., 2007; Jankovic et al., 2007). In the case of human VL, systemic IL-12 is known to be elevated, and that it induces simultaneous production of INF- γ and IL-10 by both CD4⁺ and CD8⁺ T cells (Nylen et al., 2007). Nonetheless, a study of *L. chagasi*-infected patients revealed that patients with VL presented higher numbers of CD8⁺T cells in peripheral blood than the asymptomatic or non-infected individuals (Peruhype-Magalhaes et al., 2006). Furthermore, CD8⁺T clones isolated from patients with VL produced IL-10 and suppressed effector T cells isolated from the same patient after cure (Holaday et al., 1993).

IL-10 also promotes B cell survival and plasma-cell differentiation. Indeed, polyclonal B cell activation and hypergammaglobulinemia are common in several human systemic diseases, including VL. There are several reports of autoantibody production and immune complex formation in VL, suggesting no benefits in the antibody production, except for the disease diagnosis (Sousa-Atta et al., 2002; Elshafie et al., 2007). Indeed, B cell-deficient mice have enhanced resistance to *L. donovani* infection, revealing that these cells and antibodies contribute to parasite persistence (Smelt et al., 2000). Moreover, immune complex formation induced the production of IL-10 by PBMC, mouse and human macrophages (Kane and Mosser, 2001; Miles et al., 2005; Elshafie et al., 2007). Based on this, it is possible that the production of antibodies and the formation of immune complexes drive the secretion of IL-10 from T cells to other cells, such as macrophages, as the disease progress.

Chapter III

Treatment of VL: past, present and future

The geographic distribution of leishmaniasis illustrates that it is a poverty associated disease. With the exception of southern Europe, the access to ready diagnosis, affordable treatment and effective disease control is limited due to economic reasons. Since no approved human vaccine is available, disease control is dependent on drug therapy, even though the recommended drugs are decades old, induce severe side effects, lead to the emergence of resistances and have limited efficacy due to disease exacerbation mainly associated with compromised immune capability (e.g. HIV co-infection). Greater attention should be paid to this last point, since HIV-infected patients usually develop asymptomatic visceral infections, widespread atypical organ infections, reduced responsiveness to chemotherapy and high relapse rates when the treatment is discontinued (Pintado et al., 2001; Lyons et al., 2003; Morales et al., 2002). Given all this, the available therapy is far from satisfactory and there are, according to WHO, several reasons for encouraging the discovery of new compounds or formulations which are active against leishmaniasis. However, its several clinical manifestations and the diversity of *Leishmania* species that can cause human disease dictate the impossibility of a single compound or formulation that would be effective against all forms of the disease. Thus, based on the scope of this thesis, the present chapter will focus on the visceral leishmaniasis treatment options and future perspectives.

1 Current treatment options

1.1 Pentavalent antimonials

The drugs recommended by WHO as first line treatment against cutaneous and visceral leishmaniasis belong to the pentavalent antimonial family (SbV) and were introduced over 60 years ago. This family of compounds, represented by sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucantime®), remains the mainstay therapy for leishmaniasis in most of the world's regions, with the exception of Europe and the Bihar State, India (Figure 7). Indeed, antimonial-based therapy has disadvantages that include the long term treatment requiring parenteral administration under close medical supervision due to high toxicity (arthralgia, nausea, abdominal pain; chemical pancreatitis and cardiotoxicity associated with high doses) (Guerin et al., 2002). The treatment of visceral leishmaniasis with these drugs was initially done using 10mg/kg per day for 6-10

days, but increased unresponsiveness in India led to the use of higher doses and prolonged therapy. Nowadays, and over the last few years, the State of Bihar in India has been faced with the emergence of parasite resistance to antimony therapy, where 60% of previously untreated patients are unresponsive (Lira et al., 1999; Sundar et al., 2001). Indeed, relapses after inadequate treatment with a single drug select resistant parasites which are easily spread by anthroponotic transmission (Bryceson, 2001). However, outside Bihar the treatment with pentavalent antimonials remains effective at 20mg/kg per day for 30 days.

Post kala-azar dermal leishmaniasis is frequent in India and Sudan and is commonly caused by *L. donovani*. However the Indian PKDL requires a longer term treatment (more than 120 days) while Sudanese PKDL usually requires 2 months (Thakur et al., 1990; Zijlstra et al., 2003).

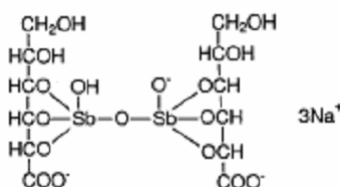


Figure 7. Chemical structures of sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucantime®).

1.1.1 Action mechanism

Even though SbV have been in clinical use for a long time, their precise action mechanism remains unknown. The first studies suggested that pentavalent antimonials inhibit the parasite glycolysis and fatty acid β -oxidation; however, no specific target on these pathways was identified (Croft et al., 2006). It is now well accepted that SbV is a prodrug that requires biological reduction to trivalent antimonials (SbIII) for antileishmanial activity (Ouellette et al., 2004; Croft et al., 2006), but the mechanism of how (enzymatic versus non enzymatic) and where (amastigote versus macrophage) it occurs remains unclear (Roberts & Rainey, 1993; Sereno et al., 1998; Shaked-Mishan et al., 2001). However, parasite specific enzymes capable of reducing the SbV to SbIII, such as the thiol-dependent - reductase 1 (TDR1) and the antimonite reductase 2 (ACR2), have been identified as potential candidates (Denton et al., 2004; Zhou et al., 2004). The non-enzymatic reduction of SbV has been attributed to parasite and macrophage specific thiols such as trypanothione and glycylcysteine, respectively (Santos Ferreira et al., 2003). Furthermore, SbIII has also been shown to inhibit trypanothione reductase

and glutathione synthetase *in vitro* (Cunningham et al., 1994; Wyllie and Fairlamb, 2006). Moreover, exposure to trivalent antimonials led to an efflux of glutathione and trypanothione from promastigotes and isolated amastigotes, suggesting an interference with the parasites' redox state (Wyllie et al., 2004). The parasite's antimonial-induced death shares phenotypical similarities with apoptosis involving DNA fragmentation and phosphatidylserine externalization (Sereno et al., 2001; Lee et al., 2002; Sudhandiran and Shaha, 2003). Besides the direct effect on the parasite, it was found that stibogluconate inhibits host cell tyrosine phosphatases, leading to an increased secretion of cytokines, suggesting therefore that the host's response is also implicated in the antimony activity (Pathak and Yi, 2001). Moreover, a recent study has demonstrated that SbV treatment of human macrophages alters the expression of only a few genes and some of their encoding proteins might be implicated in the mode of action of SbV (El Fadili et al., 2008).

1.1.2 Resistance

The mechanisms underlying the resistance to SbV therapy have been the subject of intensive research. However, most of the potentially involved mechanisms were determined using *in vitro*-selected parasite lines rather than clinical resistant isolates (Croft et al., 2006; Ouellette et al., 2004). Among the mechanisms proposed as being involved in antimony resistance is the decreased biological reduction of SbV to SbIII, observed in *L. donovani* promastigotes and axenic amastigotes upon *in vitro* selection (Shaked-Mishan et al., 2001). Furthermore, increased levels of trypanothione have been observed in some SbIII resistant lines, suggesting that increased levels of thiols such as trypanothione and glutathione could help restore thiols lost due to efflux as well as restoring thiol redox potential (Mukhopadhyay et al., 1996; Wyllie et al., 2004).

The analysis of the *Leishmania* genome has revealed eight putative homologues belonging to the MRP1 (multi-drug resistance related protein 1), known to be involved in thiol-associated efflux and metal resistance in mammalian cells (reviewed in Haimeur et al., 2004). Two of them, the multi-drug resistance related protein A (MRPA) and the pentamidine resistance protein 1 (PRP1) seem to be involved in the *Leishmania* antimony resistance (reviewed in Ashutosh et al., 2007). Resistance studies using clinical isolates are now emerging, allowing the conclusion that *Leishmania* resistance to antimony is a multifactorial phenomenon involving some of the pathways previously described by the use of *in vitro* generated resistant lines (Mandal et al., 2007; Singh et al. 2007; Mukherjee et al. 2007).

1.2 Pentamidine

Pentamidine (isethionate or methansulphonate), an aromatic diamidine, has been previously used as a second line treatment for VL (Figure 8). Even though it was initially effective in the treatment of pentavalent antimony-resistant VL, its declining effectiveness (cure rates of 70%) suggests the emergence of drug resistances (Jha et al., 1991). Moreover, its toxicity (hypotension, hypoglycemia, nephrotoxicity, irreversible diabetes mellitus and even death) has led to its complete abandonment in India (Sundar et al., 2001).

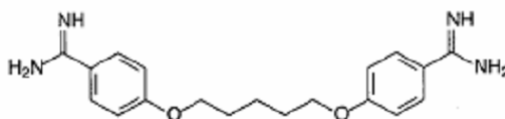


Figure 8. Chemical structure of Pentamidine.

1.2.1 Action mechanism

As in the case of pentavalent antimonials, the mechanism mediating pentamidine anti-*Leishmania* activity remains unknown. Initial studies suggested that pentamidine could enter the cell through a polyamine or arginine transporter (Kandpal and Tekwani, 1997). However, recent studies have revealed that pentamidine transport was not competitively inhibited by polyamines, aminoacids, nucleobases, sugars or other metabolites (Basselin et al, 2002). Furthermore, once inside the cell, pentamidine accumulate in the mitochondria and efficiently enhanced the efficacy of mitochondrial respiratory chain complex II inhibitors (Mehta and Shaha, 2004).

1.2.2 Resistance

Resistance to pentamidine has been generated in both promastigotes and axenic amastigotes *in vitro*. Resistant parasites presented changes in the intracellular concentrations of polyamines and arginine (Basselin et al., 2000). Moreover, in resistant clones the drug did not accumulate in the mitochondria and the cytosolic drug-containing fraction was extruded from the cell (Basselin et al., 2002). The drug efflux could be mediated by the Pentamidine Resistance Protein 1 (PRP1) which, belonging to the P-glycoprotein (PGP)/MRP ATP-binding cassette (ABC) transporter superfamily, was isolated by functional cloning while selecting for pentamidine resistance (Coelho et al., 2003).

1.3 Amphotericin B

Amphotericin B is an antifungal compound that has an effective antileishmanial activity (Figure 9). Due to the increased emergence of resistances to pentavalent antimonials in India, amphotericin B deoxycholate in a dose of 0.75-1mg/kg for 15 to 20 infusions daily or in alternate days, produced cure rates of 97%, being the drug of choice in this region (Sundar et al., 2002), even though its administration requires close medical supervision due to high toxicity (fever with rigor and chills, thrombophlebitis and occasional serious toxicities like myocarditis, severe hypokalaemia, renal dysfunction and even death) (Sundar et al., 2006). The toxic events were substantially reduced by the emergence of Amphotericin B lipid formulations (Sundar et al., 2000; Sundar et al., 2004). Indeed, these formulations allowed a targeted drug delivery to parasites, once they are preferentially internalised by the reticuloendothelial cells. Three amphotericin B lipid formulations are commercially available: liposomal amphotericin B (AmBisome®), amphotericin B colloidal dispersion (Amphocil ®) and amphotericin B lipid complex (Albacete®). AmBisome® was the first to be evaluated and it is licensed in several European countries and the USA for the primary treatment of VL.

A study conducted in Bihar comparing conventional amphotericin B (1mg/kg on alternate days for 30 days) with AmBisome® and Albacete® (both administered at 2mg/kg for 5 days) revealed comparable cure rates of 96%, 96% and 92%, respectively (Sundar et al., 2004). AmBisome® is however the best tolerated (Sundar et al., 2006). Moreover, a single dose of 7.5mg/kg of AmBisome® cures 90% of VL patients in India with minimal side effects (Sundar et al., 2003). For immunosuppressed patients a total dose of 40mg/kg AmBisome® spread over 38 days is recommended, even though relapses always occur in these patients (Sundar et al., 2002; Meyerhoff et al., 1999). Until very recently, the use of amphotericin B lipid formulations was precluded from developing countries due to their high cost. This situation may change, since WHO announced a drastic reduction in price for public healthcare systems in VL endemic areas (Chappuis et al., 2007).

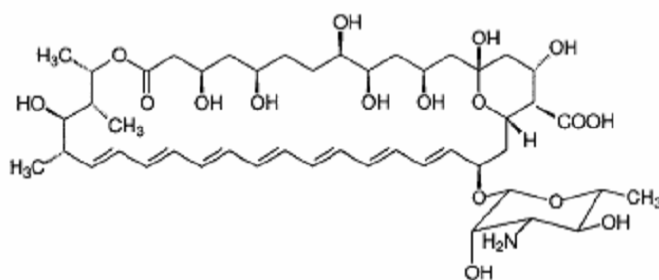


Figure 9. Chemical structure of Amphotericin B.

1.3.1 Action mechanism

The antifungal activity of Amphotericin B is attributed to its interaction with fungal membrane sterols, preferentially ergosterol. Like fungi, *Leishmania* also has ergostane-based sterols as its main membrane sterol, and this is likely to explain the increased selectivity towards the microorganism (Goad et al., 1984). However, at concentrations higher than 0.1 μ M, it induces cell lysis through the formation of aqueous pores and consequently anionic and cationic influx (Ramos et al., 1996).

1.3.2 Resistance

An amphotericin B-resistant clone of *L. donovani* promastigotes was generated *in vitro* and showed a significant change in its plasma membrane sterol profile. Indeed, in this parasite line, ergosterol was replaced by the precursor cholesta-5,7,24-trien-3 β -ol (Mbongo et al., 1998). This probably occurred due to a defect in C-24 transmethylation caused by loss of function of the S-adenosyl-L-methionine-C₂₄- Δ -sterolmethyltransferase (SCMT). The characterization of the two enzyme transcripts in the *L. donovani* promastigotes' resistant line revealed that one of them was absent and the other was overexpressed but without a spliced leader sequence, which would prevent translation (Pourshafie et al., 2004). The modification of plasma membrane sterol composition was further reported in amphotericin B resistant *L. mexicana* promastigotes and amastigotes (Hamdan et al., 2005).

Although widely used as an antifungal, resistance to amphotericin B in fungal isolates has been very rarely reported and is usually specie dependent (Ellis et al., 2002). In the case of leishmaniasis, resistance to amphotericin B was only reported in two inconclusive studies from France in the context of HIV/*L. infantum* co-infection. However, the increased use of amphotericin B and their lipid formulations in endemic areas, the treatment of HIV/*Leishmania* co-infected patients and the

insistence of some dog owners in Europe on treating *L. infantum*-infected dogs with amphotericin B lead us to think that the emergence of amphotericin B resistances could be a matter of time.

1.4 Miltefosine

Miltefosine (hexadecylphosphocholine) is an alkyl phospholipid that was initially introduced as an antineoplastic agent, later becoming the first oral antileishmanial drug to treat VL (Figure 10) (Berman, 2005). This drug was registered in India in March 2002 for the treatment of VL, is highly effective (adults and children) and well tolerated, with limited side effects that are usually mild and temporary (Sundar et al., 1998; Sundar et al., 1999; Sundar et al., 2000; Sundar et al., 2002; Sundar et al., 2003; Bhattacharya et al., 2004). The recommended therapy regimen is a single oral dose of 50mg for patients with less than 25kg body weight or a twice daily dose of 50mg over a period of 28 days for patients weighing more than 25kg (Sundar et al., 2002). However, miltefosine is teratogenic, its use being strictly forbidden in pregnant women or in women who could become pregnant within two months of treatment (Sindermann et al., 2006). A phase IV clinical trial recently conducted in India revealed that miltefosine has similar efficacy in treating VL patients in field as in hospital conditions (final cure rate was 82% of intention to treat analysis and 95% per protocol analysis similar to the 94% cure rate of hospitalized patients) (Bhattacharya et al., 2007; Sundar et al., 2002). Adverse events of common toxicity criteria grade 3 occurred in 3% of patients, including gastrointestinal toxicity and rise in aspartate aminotransferase, alanine aminotransferase, or serum creatinine levels (Bhattacharya et al., 2007). According to two different studies, one from northern Ethiopia and the other from Spain, miltefosine is less effective in HIV co-infected patients (Ritmeijer et al., 2006; Troya et al., 2008).

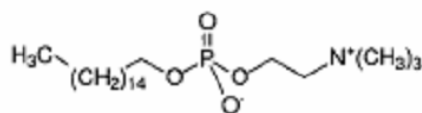


Figure 10. Chemical structure of miltefosine.

1.4.1 Action mechanism

Miltefosine induces the parasite's killing through an apoptosis-like process that involves mitochondria membrane depolarization due the inhibition of cytochrome C oxidase (Paris et al., 2004; Verma et al., 2004; Santa-Rita et al., 2004; Luque-Ortega and Rivas, 2007). Moreover, miltefosine alters the biosynthesis of a variety of lipids. Indeed, in *L. mexicana* promastigotes, it inhibited the remodelling of ether-lipid by alkyl-specific acyl coenzyme A acyltransferase (Lux et al., 2000). Furthermore, miltefosine resistant *L. donovani* exhibited changes in the length and the level of unsaturation of fatty acids, as well as a reduction in ergosterol (Rakotomanga et al., 2005)

In the context of the host cells, miltefosine does not induce NK cell activation, cytotoxic spleen cells or humoral response, but induces immunological and inflammatory effects on isolated mononuclear cells and macrophages (Hilgard et al., 1991; Beckers et al., 1994; Zeisig et al., 1995). Moreover, the leishmanicidal activity of miltefosine does not require host T cell-dependent or activated macrophage-mediated mechanisms in animal models (Murray and Delph-Etienne, 2000; Escobar et al., 2002)

1.4.2 Resistance

Despite miltefosine efficacy in the treatment of VL, its widespread use may contribute to the emergence of resistances, since resistant parasites are easily generated in laboratory and this drug has a long half-life (~150hrs) (Perez-Victoria et al., 2006). Thus, non adherence to the recommended therapy could lead to widespread parasite resistance (Thakur et al., 2000). The *in vitro* miltefosine resistance phenotype is associated to a decreased accumulation of the drug in the cell due to an increased drug efflux and a decreased drug uptake (Perez-Victoria et al., 2001; Perez-Victoria et al., 2003; Seifert et al., 2003). Overexpression of the ABC transporter, P-Glycoprotein, and inactivation of any of the proteins involved in miltefosine uptake, the transporter LdMT (included in the phospholipid translocase subfamily of P-type ATPases) and its subunit LdRos3 are mediating the increased drug efflux and the decreased drug uptake, respectively (reviewed in Perez-Victoria et al., 2006).

1.5 Paromomycin

Formerly known as aminosidine, paromomycin is an aminoglycoside antibiotic that also has antileishmanial activity (Figure 11). Even though this activity was discovered in the 1960s, it remained neglected for a long period of time, and only in August 2006 was paromomycin registered in India for the treatment of leishmaniasis (Chappuis et al., 2007). The phase III clinical trials in India revealed that paromomycin (11mg/kg/day, intramuscularly, for 20 consecutive days) is not inferior to amphotericin B (1mg/kg/day, intravenously, for 30 consecutive days) in the treatment of VL (Sundar et al., 2007). In patients receiving paromomycin no nephrotoxicity was observed, reversible damage to the inner ear was found in 2% of patients, and 1.8% of patients showed a significant increase (>fivefold) in hepatic transaminases (Sundar et al., 2007). Furthermore, paromomycin is the least expensive leishmaniasis treatment available (Chappuis et al., 2007).

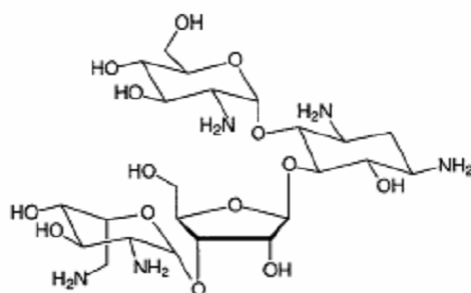


Figure 11. Chemical structure of paromomycin.

1.5.1 Action mechanism

The mechanism of how paromomycin exerts antileishmanial activity still requires further studies, although it is known that paromomycin targets *Leishmania* ribosomes, and inhibits RNA synthesis, followed by protein synthesis shown along with respiratory dysfunction (Maarouf et al., 1995; Maarouf et al., 1997).

1.5.2 Resistance

Paromomycin *L. donovani* resistant parasites were easily generated *in vitro*. The resistance showed to be specific to paromomycin and due to a decreased uptake of the drug (Maarouf et al., 1998), but further studies are required once the drug has moved to the clinical field.

2 Clinical trials

2.1 Drugs

Sitamaquine is a drug active against VL that reached clinical trials and may have future impact in the treatment of this disease. Indeed, **sitamaquine** is an orally active 8-aminoquinolone derivative whose antileishmanial activities were validated in animal models many years ago (Chapman et al., 1979; White et al., 1989). Phase II studies were conducted in India, Kenya and Brazil and the rate of efficacy ranged from 27 to 87% (Dietze et al., 2001; Jha et al., 2005; Wasunna et al., 2005). Phase IIb and III are respectively ongoing and being planned in India (Chappuis et al., 2007).

2.2 Combined therapy

The appeal to a combined therapy has been suggested as a possible way to increase treatment efficacy, prevent the development of drug resistances and reduce the treatment's duration (Bryceson et al., 2001). The efficacy and safety of a sodium stibogluconate and paromomycin combined therapy has been assessed in Kenya, Sudan and India. According to these clinical studies, the combined therapy revealed to be more effective than the therapy with sodium stibogluconate alone. Moreover, combined therapy resulted in a reduction of therapy duration (from 30 to 17-21 days) (Seaman et al., 1993; Thakur et al., 2000; Melaku et al., 2007). A combination of amphotericin B in liposomes and miltefosine is being currently evaluated in India (Chappuis et al., 2007).

Another combined therapy already subjected to clinical evaluation involved the use of rINF- γ and pentavalent antimonials. This combined therapy revealed to be useful in the treatment of severe or pentavalent antimonial-refractory VL in Brazil (Badaro et al., 1990). However, a large randomized clinical study conducted in India revealed no beneficial effect in the combination of INF- γ and pentavalent antimony (Sundar et al., 1997)

3 Drug development: future perspectives

The discovery and development of drugs for parasitic diseases have been neglected due to the lack of economic incentives. Indeed, only 1% of the new drugs introduced in the market between 1975 and 1996 were for the treatment of tropical diseases (malaria, trypanosomiasis, leishmaniasis and tuberculosis) that together contributed to 5% of the global diseases burden (Date et al., 2007). Recently, an innovative discovery strategy in drug development for tropical parasitic diseases, involving integrated partnership and networks between academic researchers and industry, has been implemented. Its main goals are to enhance cost-effectiveness and increase the chance of success (Nwaka and Hudson, 2006). Indeed, new treatments for tropical parasitic diseases could be discovered following short term approaches [including the combination of available commercial drugs (mentioned in the previous section), the development of new formulations for available drugs, and new applications for existing drugs] or long term approaches (discovery of new molecules).

3.1 *In vitro* and *in vivo* assays

In the search for new drugs or formulations with antileishmanial activity, specific *in vitro* and *in vivo* assays are needed. The requirements of the *in vitro* assays to evaluate the compounds' intrinsic antileishmanial activity include the use of: parasite mammalian stage, a dividing population, and quantifiable and reproducible measurements of the drug's activity (Croft et al., 2006). Indeed, drug screening assays using promastigotes are easy to perform, but significant biochemical differences exist between this parasite stage and the relevant stage (amastigotes) (Carrio et al., 2000; Agnew et al., 2001). However, the possibility to culture axenic amastigotes of some *Leishmania* spp. overcome that limitation (Bates et al., 1994; Balanco et al., 1998; Sereno et al., 1997; Ephros et al., 1999). Drug screening using such parasite forms can be achieved by: microscopy parasite count (Callahan et al., 1997), MTT based assays (Ganguly et al., 2005), fluorescence-activated-cell-sorter (FACS) and propidium iodide (Sereno et al., 2005; Vergnes et al., 2005) and more recently, by the use of parasites transfected with reporter genes (Naylor et al., 1999). Indeed, parasites transfected with reporter genes encoding enzymes such as luciferase, β -galactosidase or β -lactamase represent a meaningful tool to determine drug efficacy in intracellular amastigotes, since these determinations were classically performed by direct counting, which is very laborious, time

consuming and may give inaccurate determination of the IC₅₀, given the difficulty in evaluating parasite viability by a staining procedure (reviewed in Sereno et al., 2007). Meanwhile differences in drug sensitivity between axenic and intracellular amastigotes were already reported, supporting the necessity of evaluating drug efficacy against the intracellular forms (Ephros et al., 1999).

The Balb/c mice model is commonly used to perform *in vivo* experiments to evaluate drug efficacy in VL. However it is necessary to characterize the infection in each mice model to assure that the drug is tested appropriately. In the case of VL, a common problem in all mice models is related with the evaluation of the drug activity requiring biopsies or necropsy and further microscopy analysis. PCR techniques have been developed to give a more accurate evaluation of the parasite load (Mary et al., 2004; Prina et al., 2007).

3.2 Development of new formulations

The ability of *Leishmania* parasites to survive and multiply inside macrophages also protects the parasite from the action of drugs that have difficulties in penetrating phagocytic cells. Such drug difficulties can be overcome by the use of delivery systems capable of selective distribution in phagocytic cells. Additionally, these systems may prevent the broad distribution of drugs throughout the body and their presence in uninfected tissues, which, besides its inherent toxicity, will induce side effects. Liposomes, nanosuspensions, polymeric and lipid nanoparticles are examples of such delivery systems. The carrier's surface can be modified by the binding of ligands which are recognized by specific receptors of phagocytic cells, thereby facilitating their internalization through receptor-mediated endocytosis. Mannosyl/fucosyl receptors and macrophage scavenger receptors are the most studied ones (Mukhopadhyay and Basu, 2003; Vasir et al., 2005).

The potential of delivery systems in the treatment of leishmaniasis is sustained by the efficacy of amphotericin B in liposomes (AmBisome®). Liposomes are the most studied colloidal carriers. Indeed, they are microscopic vesicles consisting of one or more concentric spheres of lipid bilayers separated by an aqueous compartment whose diameter ranges from 80nm to 100µm. Among the several modifications introduced in liposomes to increase drug delivery to macrophages that improved drugs antileishmanial activity are: sugars (Owais et al., 2005) such as mannose (Banerjee et al., 1994; Banerjee et al., 1996; Sinha et al., 2000; Basu et al., 2004; Mitra et al., 2005), phosphatidylcholine and stearylamine, that conferred positive charges to liposomes (Miller et al., 1998; Dey et al., 2000; Pal et al., 2004; Banerjee et al., 2008), peptides such as tuftsin (Guru et al., 1989) and

the chemotactic peptide, f-Met-Leu-Phe (fMLP) (Barjee et al., 1998), and antibodies (Dasgupta et al., 2000; Mukherjee et al., 2004).

Moving to the nanoparticulate delivery systems (polymeric nanoparticles, solid lipid nanoparticles and lipid drug conjugates), these have been extensively studied in the last few years. Their main advantage when compared to liposomes is the ability to withstand physiological stress or improved biological stability and the possibility of oral delivery (Lockman et al., 2002; Couvreur et al., 2006). The polymeric nanoparticles are solid colloidal particles (size 1-1000nm) made of biocompatible polymers in which the compound can be adsorbed, entrapped or covalently attached (Lockman et al., 2002). Drugs containing nanoparticles made of different polymers including polyalkylcyanoacrylate (PACA) (Gaspar et al., 1992), polylactic acid (PLA) (Rodrigues et al., 1994), polymethyl methacrylate (Fusai et al., 1997; Paul et al., 1998) and the biodegradable poly(ϵ -caprolactone) (Espuelas et al., 2002) were tested against experimental leishmaniasis, increasing drug effectiveness. Indeed, polymer hydrophobicity seems to be a major factor governing macrophages uptake, since as an example, nanoparticles made of polymethyl methacrylate target macrophages better than the ones made of polyalkylcyanoacrylate (Basu et al., 2004).

3.3 New indications for existing drugs

Extending drug indications beyond those for which they were developed is a strategy for increasing the drug repertoire of a given disease, saving time and money. Indeed, this approach has been very well succeeded in the field of parasite diseases (Pink et al., 2005). In the case of leishmaniasis, the most recent example is the registration in India of the anticancer miltefosine as an antileishmanial drug. Indeed, several anticancer drugs have shown activity against both cancer and *Leishmania* (Miguel et al., 2008; review in Fuertes et al., 2008). Another example are bisphosphonates, namely risedronate and pamidronate, which are commonly used to treat bone disorders, but were also active against experimental CL and VL (Rodriguez et al., 2002; Yardley et al., 2002).

3.4 Discovery of new molecules

The discovery of new molecules could be achieved by screening compound libraries using whole parasites or, instead, using a previously validated parasite target. The high-throughput screening of compound libraries using whole parasite is gaining new relevance in *Plasmodium*, *Trypanosome* and *Leishmania* spp. due to the possibility of obtaining such parasites transfected with reporter genes (Pink et al.,

2005). Among the screened libraries of compounds are the ones made of natural products (reviewed in Tagboto et al., 2001).

The target-based drug discovery is a very expensive and time consuming rational approach, but it permits increased knowledge of parasite biology. The identification and validation of new targets is mostly supported, nowadays, by the genomics programs, genetic knockout techniques and RNA interference (RNAi). The selection of a target based on genomics screening implies its validation by genetic or chemical approaches. Moreover, the target should be biochemical and structurally characterized, subject to selective inhibition without developing resistances, and technically accessible to the screening of several compounds (Pink et al., 2005). Different laboratories have engineered genetically defined mutant *Leishmania* parasites, aimed at identifying parasite virulence or disease persistence factors that could allow the identification of either potential drug targets or attenuated live vaccines. Some of the mutants showing attenuated virulence are lacking: dihydrofolate reductase-thymidylate synthase (DHFR-TS) in *L. major* (Cruz A et al., 1991; Titus RG et al., 1995), cysteine protease in *L. mexicana* (Souza AE et al., 1994; Mottram JC et al., 1996; Alexander J et al., 1998), biopterine transporter in *L. donovani* (Papadopolou B et al., 2002), LPG2 in *L. major* (the gene encoding Golgi GDP-Mannose transporter) (Spath GF et al., 2003; Uzonna JE et al., 2004), glutamine:fructose-6-phosphate amidotransferase in *L. major* (Naderer et al., 2008) and delta subunit of the adaptor protein 3 in *L. major* (Besteiro et al., 2008). The progresses in the characterization of some of the above-listed proteins' potential as drug targets and the search for inhibitors are mentioned on the review paper included in this dissertation.

Therapy and Further Development of Anti-Leishmanial Drugs

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Abstract: Leishmaniasis is a parasitic infection that affects millions of people worldwide, especially in tropical and sub-tropical areas, and is responsible for high mortality and morbidity. The therapies available up to the present are far from satisfactory and, since leishmaniasis affects poor people in poor regions, the development of new drugs has been neglected due to the lack of commercial motivation. Safe and orally available drugs, especially against the visceral form of the disease, are needed. An overview of the main strategies for antileishmanial drug development, mainly focused on the target-based drug development approach, is given.

Key Words: *Leishmania*, drugs, targets.

INTRODUCTION

The protozoan parasite *Leishmania*, the agent of leishmaniasis, is responsible for serious mankind diseases in tropical and subtropical areas of the world. They are characterized by diverse clinical manifestations varying from localized ulcerative skin lesions and destructive mucosal inflammation to disseminated visceral infection, also known as kala-azar. The geographic distribution of leishmaniasis illustrates that it is a poverty-associated disease. 90% of the cutaneous cases occur in Afghanistan, Pakistan, Syria, Saudi Arabia, Algeria, Iran, Brazil and Peru, and 90% of the visceral disease occurs in India, Bangladesh, Nepal, Sudan and Brazil [1]. Except in southern Europe, the access to ready diagnosis, affordable treatment and effective disease control is limited due to economic reasons. Visceral leishmaniasis (VL), caused by *Leishmania infantum*, is a serious problem throughout the Mediterranean basin, where dogs are the most important reservoirs of the parasite. In humans, during the last few years, VL has been mainly associated with HIV co-infection and regarded as an emerging disease, with 9% of AIDS patients suffering from newly acquired or reactivated visceral leishmaniasis [2]. A vertebrate host becomes infected by *Leishmania* after the bite of the sandfly (*Phlebotomus* and *Lutzomyia* spp) during a blood meal, through the inoculation of infective flagellated promastigotes that invade or are phagocytosed by local or recruited host cells. In the phagolysosomes, the promastigotes will differentiate into non flagellated amastigotes that multiply and are able to infect other adjacent or distant macrophages. Disease control is dependent on drug therapy, since no approved vaccine is available. The available drugs are decades old, induce severe side effects and the emergence of resistances, and have limited efficacy due to disease exacerbation mainly associated with compromised immune capability. Greater attention should be paid to this last point, since HIV-infected patients usually develop asymptomatic visceral infections, wide-

spread atypical organ infections, reduced responsiveness to chemotherapy and high relapse rates when the treatment is discontinued [3-5]. Given that the available therapy is far from satisfactory, there are, according to the World Health Organization (WHO), several reasons for encouraging the discovery of new compounds or formulations which are active against leishmaniasis.

CURRENT DRUG THERAPY

The drug repertoire to treat leishmaniasis is to date very small, in spite of recent efforts to reverse this situation. Introduced 60 decades ago, the drugs recommended by WHO as first line treatments against cutaneous and VL belong to the pentavalent antimonial family and are represented by sodium stibogluconate (Pentostam, SSG) and meglumine antimoniate (Glucantime). These remain the cornerstone drugs in all regions except two: Bihar State in India, where resistance is up to 60%, and southern Europe, where patients are usually treated with the liposome amphotericin B, which is effective in a short-course treatment [6-8]. The emergence of resistances in Bihar, India, is mainly associated with irregular drug use and incomplete treatment [9], therefore *Leishmania/HIV* co-infected individuals may constitute a potential source of resistant strains due to their slow response to treatment and weak immune response [10]. Amphotericin B revealed itself to be active not only against fungal infections but also against *Leishmania* and *Trypanosoma cruzi* infections. The amphotericin B deoxycholate (Fungizone®) is a second line treatment, except in Bihar State in India, where it is a first line drug. This drug's main drawback is that it has the potential to induce acute toxicity requiring patient hospitalization. The search for new formulations in order to avoid toxicity has led to the implementation of a very active and effective formulation against VL, amphotericin B in a liposome formulation, (AmBisome®), limited to Europe due to its high cost [7]. Miltefosine, an anticancer drug, was recently registered in India (2002) for oral treatment of VL and in Colombia (2005) to treat cutaneous leishmaniasis (CL). It has many advantages, including the short course of treatment, oral efficiency and low cost. On the other hand, concerns about teratogenicity and the long

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half-life of the drug ($t_{1/2} = 7$ days), that could favour the development of resistances, are the main problems associated with its use [11].

Table 1. Drugs for the Treatment of Leishmaniasis

Current Therapy	Sodium Stibogluconate (Pentostam) Meglumine antimoniate (Glucantime) Amphotericin B (Fungizone) Liposomal Amphotericin B (Ambisome) Miltefosine (India and Colombia)
Clinical Trials	Paromomycin Imiquimod Sitamaquine

DRUGS IN CLINICAL TRIALS

Even though many compounds have been described as active *in vitro* against *Leishmania*, just a few have been tested and shown to be active in rodent models. Consequently, it is easy to summarize the number of molecules, which, in clinical trials, are active against leishmaniasis. Belonging to the aminoglycoside antibiotic family, paromomycin is active against leishmaniasis and has reached the clinical trials for both VL and CL. In very recent, randomized controlled phase 3 open-label studies conducted in Bihar, India, the efficacy of paromomycin to treat VL was compared with amphotericin B. This study revealed that in India, paromomycin is not inferior in the treatment of VL when compared to amphotericin B (final cure rate, 94.6% vs. 98.8%) [12], though the occurrence of adverse reactions was more common in patients receiving paromomycin than in patients receiving amphotericin B (6% vs. 2%) [12]. The possible treatment of CL with topical formulations containing paromomycin evaluated in clinical trials revealed variable efficacy that could be attributed to the formulation, the type of lesion being treated and the strain of *Leishmania*-induced disease [13-15]. Another compound in clinical trials for the treatment of leishmaniasis is imiquimod, an immunomodulator used in clinics to treat human papillomavirus that induces genital infections. This compound is capable of stimulating a local immune response, suggesting its potential application in several situations. Able to activate the macrophage leishmanicidal activity, imiquimod became attractive for use in the treatment of CL [16]. Even though the combination of imiquimod and antimonials has produced good results in the treatment of CL in patients who had not responded to the antimonial therapy alone, a recent study has shown no benefits in the combined therapy of imiquimod and meglumine antimoniate in patients with CL in an endemic area of *L. tropica* [17,18].

Sitamaquine is an 8-aminoquinolone with oral bioavailability that has been included in clinical trials to treat VL in Brazil, India and Kenya, with a success level ranging from 67 to 92% cure rate [19-21].

FUTURE PERSPECTIVES FOR DRUG DEVELOPMENT

An innovative discovery strategy in drug development for tropical parasitic diseases, involving integrated partner-

ship and networks between academic researchers and industry, has been implemented. Its main goals are to enhance cost-effectiveness and increase the chance of success [22]. New treatments for tropical parasitic diseases could be discovered following several approaches, including the combination of available commercial drugs, the discovery of new applications for existing drugs and the discovery of new molecules. The latter could be achieved by screening compound libraries using whole parasites or, instead, using a previously validated parasite target. From among the strategies mentioned, we will focus on the target-based drug discovery. This rational approach is both very expensive and time consuming, but it permits the increase of knowledge of parasite biology. The identification and validation of new targets is mostly supported, nowadays, by the genomics programs, genetic knockout techniques and RNA interference (RNAi). The selection of a target based on genomics screening implies its validation by genetic or chemical approaches. Moreover, the target should be biochemical and structurally characterized, subject to selective inhibition without developing resistances, and technically accessible to the screening of several compounds [23]. Different laboratories have engineered genetically defined mutant *Leishmania* parasites, aimed at identifying parasite virulence or disease persistence factors that could allow the identification of either potential drug targets or attenuated live vaccines. Among the several mutants, we will focus on the ones which not only showed attenuated virulence *in vivo* but also conferred protection against a challenge with virulent parasites, and record the efforts made as potential drug targets. Protection from virulent challenges in mice was reached with mutant parasites carrying deletions in the following genes: dihydrofolate reductase-thymidylate synthase (DHFR-TS) in *L. major* [24, 25], cysteine protease in *L. mexicana* [26-28], bipterine transporter in *L. donovani* [29] LPG2 in *L. major* (the gene encoding Golgi GDP-Mannose transporter) [30, 31] and SIR2 in *L. infantum* (the gene encoding a silent information regulatory 2 homologue protein) [32, 33].

Dihydrofolate Reductase Thymidilate Synthase

The bifunctional enzyme dihydrofolate reductase thymidilate synthase (DHFR-TS), which has a role in folate reduction and therefore thymidine biosynthesis, is an interesting target for the development of drugs against protozoa parasites [24]. Among these, significant differences between the protozoa and the human enzyme, in which the DHFR and the TS occur as two separate monofunctional proteins, potentiality reinforce it as a drug target [34]. In fact, inhibitors of DHFR have been very successful in the treatment of several diseases like malaria (pyrimethamine and cycloguanil), bacterial infections (trimethoprim) and cancer (methotrexate). The absence of such success in the case of *Leishmania* and *Trypanosome* infections seems to be due to pteridine reductase 1 (PTR1), that can perform folate reduction when endogenous DHFR is inhibited [35]. However, the crystallography of DHFR from *L. major* permitted the detection of structural differences between the parasitic and the human enzyme that could be exploited to design selective inhibitors [36]. Only a few molecules that selectively inhibit the leish-

manial DHFR are described in the literature. The substituted 5-benzyl-2, 4-diaminopyrimidines, and particularly the 8-octyloxy derivative, showed good activity and selectivity against the leishmanial enzyme and a low IC₅₀ against the *L. major* promastigote and *L. donovani* amastigotes [37]. Structural activity relation studies revealed that the most selective and potent alkyl-substituted 5-benzyl-2, 4-diaminopyrimidines against the *Leishmania* and *Trypanosoma* DHFR were the ones with a chain length containing 2 to 6 carbon atoms. These compounds exhibited good selectivity and activity against the parasitic enzyme, particularly to *T. brucei* when compared to the human enzyme, and were also capable of efficiently inhibiting the parasite's growth [38]. A reasonable inhibition of DHFR isolated from *L. mexicana* promastigotes and a very potent activity against *L. major* amastigotes cultured in human macrophages were found with some 2, 4-diaminoquinazolines. However, a lack of correlation between the effect on the amastigotes and the DHFR inhibition was found, suggesting an alternative mode of action on the parasite [39, 40]. Since these compounds were active *in vivo*, increasing the half-life of the mice infected with *T. cruzi*, a virtual library of 2, 4-diaminoquinazolines was created to fit the enzyme active site. The most promising compounds were synthesized and showed good inhibition against the leishmanial enzyme, but an unexpected lower activity against *L. donovani* amastigotes [41].

Cysteine Proteases

Targeting proteases are a very successful strategy in the case of the human immunodeficiency virus (HIV) and anti-hypertensive therapy. As for *Leishmania* infection, the cysteine proteases were not essential to parasite survival but do play a role in *Leishmania* infection and pathogenicity [26, 27, 42]. There are three classes of cysteine proteases, namely A (cathepsin L-like proteases), B (papain like enzymes), and C (cathepsin B-like proteases). These classes of enzymes also play a role in the host-parasite interaction, since the product of gene B 2.8 is a potent inducer of the Th2 response in Balb/c mice [43]; the cysteine proteinase B inhibits the Th1 response in C3H and C57Bl6 mice [44]. Several strategies have been developed to discover new cysteine protease inhibitors with antiparasitic activity. From the *Leishmania* targets mentioned, the cysteine proteases are the most thoroughly studied concerning inhibitor development, among the number of molecules described in the literature. The aziridine-2-3-dicarboxylate derivatives [45-47], the vinyl sulfones, the hydrazides and the thiosemicarbazones [48, 49] are examples of some classes of compounds that inhibit the parasitic cysteine proteases. More recently, the *in silico* screening methodology based on homology models allowed the identification of novel *Leishmania* cysteine protease inhibitors [50].

Biopterine Transporter

Due to the *Leishmania* auxotrophy to pterine and folates, a number of folate transporters [51] and a pterine transporter [52, 53] play an important role in cell biology. The pterines are essential for the *Leishmania*'s growth and its level is important for the parasite metacyclogenesis [54]. The genetic

inactivation of the biopterine transporter in *L. donovani* produced an attenuated strain capable of conferring protection against a challenge with a virulent strain in mice [29]. However, no reports were found in the literature concerning the development of molecules active against the *Leishmania* biopterine transporter.

Golgi GDP-Mannose Transporter

The *Leishmania* parasites are covered by a dense glycocalyx that is formed by glycosylphosphatidyl inositol, anchored to surface glycoconjugates, including lipophosphoglycan (LPG), glycoinositol phospholipids (GIPLs), and proteins, such as proteophosphoglycan (PPG) or gp63. The LPG has, in *L. major*, an important role in the establishment of infection on macrophages after metacyclic invasion [55] and depends on the Golgi GDP-mannose transporter, LPG2, (involved in the assembly of repeated units of phosphoglycan) to survive inside the macrophages [56]. The *L. major* LPG2 null mutant parasites are compromised in the induction of acute pathology, and confer protection against a challenge with a virulent parasite strain in the mice model [31]. Even the *Leishmania* LPG2 recombinant protein has already been purified and reconstituted in liposomes, showing GDP-mannose transporter activity [57]. No data were found in the literature concerning the development of inhibitors.

Silent Information Regulatory 2 Homologue

Mutant parasites of the Silent Information Regulatory 2 homologue (SIR2) were produced and characterized by our team, using a gene targeted disruption strategy [32]. The necessity of episomal rescue to achieve null chromosomal mutants (*LiSIR2*^{-/-}) as the marked disruption of the ability of the single knockout (*LiSIR2*^{+/-}) amastigotes to multiply, states the importance of the cytosolic *Leishmania* SIR2 homologue protein in the parasite's survival [32]. The fact that the single mutant parasites (*LiSIR2*^{+/-}) were able to persist in the Balb/c mice for up to six weeks but failed to establish an infection, supported the development of protective experiments towards a challenge with virulent *L. infantum*. As expected, the vaccination with live *LiSIR2* single mutant parasites (*LiSIR2*^{+/-}) provided complete protection in mice [33]. The proteins belonging to this family are being found in a variety of organisms ranging from bacteria to humans, and share a conservative core domain responsible for their deacetylase activity [58, 59]. All proteins couple the deacetylase reaction with the NAD⁺ hydrolysis, transferring the acetyl group from the substrate to ADP-ribose and consequently generating a new O-acetyl-ADP-ribose compound, whose biological function(s) remains unclear [60, 61]. Several attractive functions have been attributed to the members of the SIR2 family, including transcriptional repression, recombination, cell division, microtubule organization, cellular responses to DNA-damaging agents and an involvement in longevity, associated with caloric restriction regimens in several organisms like *C. elegans*, yeast and flies [62]. We have recently characterized and expressed a functional recombinant *LiSIR2RP1* enzyme (Tavares J. *et al.*, submitted). Studies are in progress to screen potential compounds, which could efficiently inhibit the *LiSIR2RP1* enzymatic activity.

CONCLUSIONS

Several reasons encourage the discovery of new treatments against protozoa infections. Among the strategies to find new treatments for leishmaniasis, the discovery of new applications for existing drugs has been more fruitful, when compared to the new molecules discovery approach. On the other hand, new effective molecules are urgently needed, due to the emergence of resistances and the limited efficacy of the available therapy in the *Leishmania*/HIV co-infection.

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Chapter IV

The Silent Information Regulator 2 (SIR2) family of proteins

The proteins belonging to the Silent Information Regulator 2 (SIR2) family, also known as Sirtuins, are present in a variety of organisms ranging from bacteria to humans. Their involvement in the regulation of a number of biological processes such as heterochromatin formation, gene silencing, DNA repair, development, longevity, metabolism, adipogenesis, and apoptosis has been described, attracting great interest in several research fields, including Parasitology.

1 A brief history of Sirtuins

The yeast SIR2 protein (*Saccharomyces cerevisiae*) is the founding member of the large and diverse SIR2 family of proteins. This protein was discovered while trying to understand how the yeast cell type, known as mating type, is regulated. The mating type in *S. cerevisiae* is determined by a single locus known as *MAT*, which can contain either of two alleles, *MATa* or *MAT α* . Diploid cells *MATa*/ α are only generated by the mating of the *MATa* cells with *MAT α* . Moreover, the yeast also contains in the same *MAT* chromosome, but in a different locus, two silent copies of the mating-type information known as *HMRa* and *HML α* (collectively known as *HM* loci), which encode the *a* and α information, respectively, but are transcriptionally silent. The *SIR2* gene was identified in a spontaneous mutant, with a genetic defect in maintaining the silent state of the *HM* loci (Klar et al., 1979). The interpretation of the phenotypes exhibited by the SIR2 mutants led not only to the understanding of how mating type is regulated in yeast, but also in establishing the phenomenon of transcription silencing.

Mutational studies indicated that lysine 16 in the amino-terminal tail of histone H4, and lysines 9, 14 and 18 in histone H3 are critically important in the silencing (Braunstein et al., 1993; Thompson et al., 1994; Hecht et al., 1995). Moreover, lysines 9 and 14 in histone H3, and lysines 5, 8 and 16 in histones H4 are acetylated in active chromatin and hypoacetylated in silenced chromatin (Braunstein et al., 1993; Braunstein et al., 1996). Indeed, when deacetylated, the histones can fold into a more compact nucleosomal structure (Luger et al., 1997). The global deacetylation of histones that occurred when the yeast SIR2 protein was overexpressed, suggested SIR2 to be a histone deacetylase (Braunstein et al., 1993). Furthermore, an involvement of the yeast SIR2 in longevity was also described, based on the longer life span displayed by the cells carrying extracopies

of SIR2 protein, in contrast with the reduced replicative life span of the cells lacking SIR2 (Kaeberling et al., 1999). Initial enzymatic studies with Sirtuins, performed in *Salmonella typhimurium*, revealed that the bacterial SIR2 protein, CobB, was capable of partially compensating the loss of CobT in the cobalamin biosynthesis, thus indicating ADP-ribosyltransferase as the enzymatic activity catalyzed by these enzymes (Tsang et al., 1998). Indeed, during the biosynthesis of cobalamin, CobT catalyzes the transfer of phosphoribose from nicotinic acid mononucleotide to dimethylbenzimidazole to form dimethylbenzimidazole-5'-ribosyl-phosphate (Trzebiatowski et al., 1994; Trzebiatowski and Escalante-Semerena, 1997). Even though ADP-ribosyltransferase activity was further described in eukaryotic Sirtuins (Frye et al., 1999; Tanny et al., 1999), the main enzymatic function of these enzymes was disclosed by Imai and colleagues in 2002, as being the NAD⁺-dependent deacetylase of lysines 9 and 14 of histones H3, and specifically lysine 14 of histones H4. In fact, the authors described that this enzymatic activity accounts for silencing, suppression of recombination, and life span extension (Imai et al., 2002).

2 The family of the histones deacetylases

Chromatin remodelling between relatively "open" and "closed" forms play a key role in epigenetic regulation of gene expression. Modifications in the amino terminal tail of histones are involved, and can occur by acetylation, methylation, phosphorylation, poly-ADP ribosylation, ubiquitinylation, sumoylation, carbonylation and glycosylation (reviewed in Nightingale et al., 2006). The level of histones acetylation tightly controls gene expression, and depends on the balance between the activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs) (reviewed in: Roth et al., 2001; Thiagalingam et al., 2003). The transference of acetyl groups to amino-terminal lysine residues in histones, catalyzed by HAT, results in chromatin expansion and increased accessibility of regulatory proteins to DNA (Roth et al., 2001), while the removal of these acetyl groups by HDACs led to chromatin condensation and transcriptional repression (Thiagalingam et al., 2003). Therefore HDACs deacetylate histones and certain nonhistones proteins. Eighteen HDACs that have been identified in humans were subdivided into four classes, according to their homology with the yeast proteins, subcellular localization and enzymatic function (Thiagalingam et al., 2003). Indeed, the HDACs belonging to class I are 1, 2, 3 and 8. They are homologues of the yeast RPD3 and present nuclear localization; the HDACs 4, 5, 6, 7, 9 and 10 are included in class II and

share homology with the yeast Hda1. The proteins from this family commonly shuttle between the nucleus and cytoplasm. The presence of two catalytic domains in the HDAC 6 and 10 led to an additional subdivision in classe IIB. Class I and II HDACs are zinc-dependent amidohydrolases while class III enzymes require NAD^+ for catalysis and produce nicotinamide as well as O-acetyl-ADP-ribose as consequence of acetyl transfer. Therefore class III includes the yeast SIR2 homologues that require NAD^+ for their enzymatic activities (SIRT1-7). HDAC11 is the only member of class IV and shares homology with the catalytic core domain of classes I and II (Gao et al., 2002).

Phylogenetic analysis of eukaryotic and prokaryotic conserved domains of Sirtuins allowed their organization into five main classes (I, II, III, IV and U) (Frye, 2000). However, several modifications to this classification have been proposed, since there is no obvious relation between the members and their biological role.

3 Enzymatic reactions catalyzed by Sirtuins

3.1 NAD^+ -dependent deacetylase

The catalysis of NAD^+ -dependent deacetylation reactions is well established for a number of Sirtuins. While HDACs from classes I and II catalyze the deacetylation reactions using only water as cosubstrate with favorable free energy change and a constant that favors the products, the requirement of NAD^+ by Sirtuins to remove the acetyl moiety from the ϵ amino group of lysine residues within protein targets revealed to be thermodynamically and kinetically unfavorable (Imai et al., 2000; Landry et al., 2000; Smith et al., 2000; Hernick and Fierke, 2005; Sauve et al., 2006). Indeed, the deacetylation reaction requiring NAD^+ produces the deacetylated protein, nicotinamide and O-acetyl-ADP-ribose (OAADPr) (Figure 12) (Tanner et al., 2000; Tanny et al., 2001; Sauve et al., 2001). Several questions have been raised to help understand the NAD^+ dependency of Sirtuins. Among them is the connection of the Sirtuins' activity with the cellular energy, redox and metabolic status or, alternatively, the synthesis of OAADPr as second messenger metabolite.

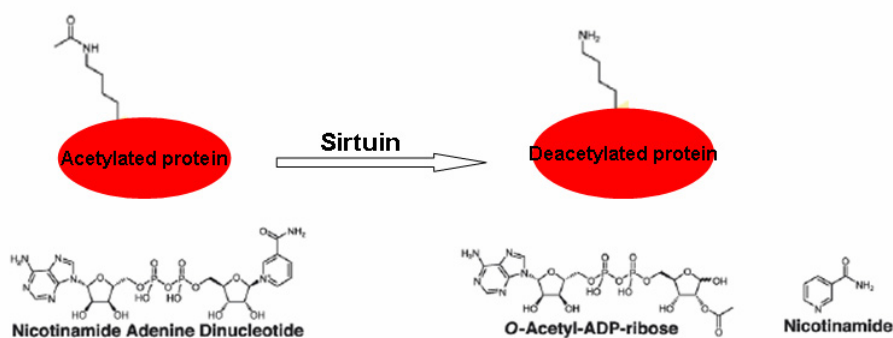


Figure 12. Sirtuin catalyzed protein deacetylation (Adapted from Milne and Denu, 2008).

The biological role of OAADPr as an effector molecule remains elusive. It has been suggested that OAADPr might be a substrate for other linked enzymatic processes, an allosteric regulator, or a second messenger. The first evidence of its potential biological activity came from the observation that the microinjection of OAADPr in starfish oocytes or blastomeres causes a block/delay in maturation and cell division, respectively (Borra et al., 2002). Furthermore, a role has been attributed to OAADPr in gene silencing, through its binding to the histone variant macroH2A1.1 found in heterochromatin regions (Kustatscher et al., 2005), or through its binding to the SIR2/3/4 silencing complex in yeast (Liou et al., 2005). The existence in the cells of enzymes capable of metabolizing the OAADPr, such as some members of the Nudix family of ADPr hydrolases, supports their role as second messengers (Rafty et al., 2002). Furthermore, OAADPr binds and activates the transient receptor potential melastatin-related channel 2 (TRPM2 channel), which is a non-selective cation channel, that under prolonged activation by oxidative and nitrative agents leads to cell death (Grubisha et al., 2006).

The NAD⁺-dependent deacetylation catalyzed by Sirtuins also involves the generation of nicotinamide. Nicotinamide is a noncompetitive (versus both NAD⁺ and acetyllysine substrates) inhibitor of Sirtuins (Landry et al., 2000; Bitterman et al., 2002; Sauve et al., 2003). In fact, nicotinamide inhibits these enzymes by interacting with a reaction intermediate via a base-exchange reaction to reform NAD⁺. The positively charged O-alkyl-amidate intermediate and nicotinamide are generated after the nucleophilic attack of the acetyl-lysine carbonyl oxygen on the C1' to the nicotinamide ribose of NAD⁺ (Sauve et al., 2001; Denu et al., 2003; Sauve and Schramm, 2004). When nicotinamide binds to the enzyme containing the O-alkyl-amidate intermediate, both can react in a process known as nicotinamide exchange, where the NAD⁺ and the acetylated peptide are reformed (Sauve et al., 2001; Jackson et al., 2003; Sauve and Schramm, 2003). In the

presence of high concentrations of nicotinamide, this reaction occurs at the expense of deacetylation.

3.2 ADP- ribosyltransferase

A number of reports have suggested that Sirtuins catalyze ADP-ribosylation either exclusively or in conjunction with their deacetylase activity. In addition to CobB other Sirtuins, like the human SIRT2 and the yeast SIR2, revealed to be capable of transferring ADP-ribose to BSA or histones (Tanny et al., 1999; Frye et al., 1999). Even though the majority of Sirtuins present robust NAD^+ -dependent deacetylase, no significant histone NAD^+ -dependent deacetylase was detected in the case of the human SIRT4 and SIRT6 (North et al., 2003; Haigis et al., 2006). Indeed, SIRT4 was shown to ADP-ribosylate and down-regulate glutamate dehydrogenase, and has been implicated in insulin regulation (Haigis et al., 2006; Ahuja et al., 2007). SIRT6 is involved in DNA base-excision repair and can mediate auto-ADP-ribosylation (Liszt et al., 2005). Moreover, yeast SIR2, Hst2 and SIR2 orthologues from protozoan parasites such as *Trypanosoma brucei* and *Plasmodium falciparum* exhibited both NAD^+ -dependent deacetylase and ADP-ribosyltransferase activities (Imai et al., 2000; Tanner et al., 2000; Garcia-Salcedo et al., 2003; Merrick et al., 2007).

Three possible mechanisms have been proposed to explain the ADP-ribosylation reaction catalyzed by Sirtuins (Figure 13) (Denu et al., 2005). One of them does not require an acetylated substrate, and involves the direct transference of the ADP-ribose moiety of NAD^+ by Sirtuins (Figure 13, A). Indeed this mechanism was proposed to occur in the case of SIRT4 and SIRT6 (North et al., 2003; Haigis et al., 2006). An alternative mechanism requires the involvement of an acetylated substrate and NAD^+ to originate the ADP-ribose-acetyl-intermediate, which is formed in the initial catalytic step of the deacetylation reaction (Sauve et al., 2001; Jackson et al., 2003; Smith et al., 2006). This intermediate is susceptible to catalytic attack by the acetylated substrate itself (*cis*) or by an acceptor protein (*trans*) (Figure 13, B) (Smith et al., 2006). In the third proposed mechanism, the enzymatic activity of Sirtuins is required only to perform NAD^+ -dependent deacetylation, since it is the reaction product OAADPr or its hydrolyzed product, ADPr, which can react non-enzymatically with an acceptor protein, resulting in ADP-ribosylation (Figure 13, C).

A recent study conducted with *T. brucei* SIR2 related protein 1 (TbSIR2rp1), as a model of Sirtuins that display both NAD^+ -dependent deacetylase and ADP-ribosyltransferase activities, has shed light on the mechanistic relationship that

exists between both enzymatic reactions (Kowieski et al., 2008). Biochemical studies have revealed that ADP-ribosylation of histones/protein mediated by TbSIR2rp1 required an acetylated substrate (Kowieski et al., 2008). Indeed, the two above-mentioned distinct pathways of ADP-ribosylation involving an acetylated substrate were supported by the experimental data obtained by Kowieski and colleagues, 2008. However, under identical conditions, the sum of both ADP-ribosylation reactions was ~ 5 orders of magnitude slower than histone deacetylation (Kowieski et al., 2008).

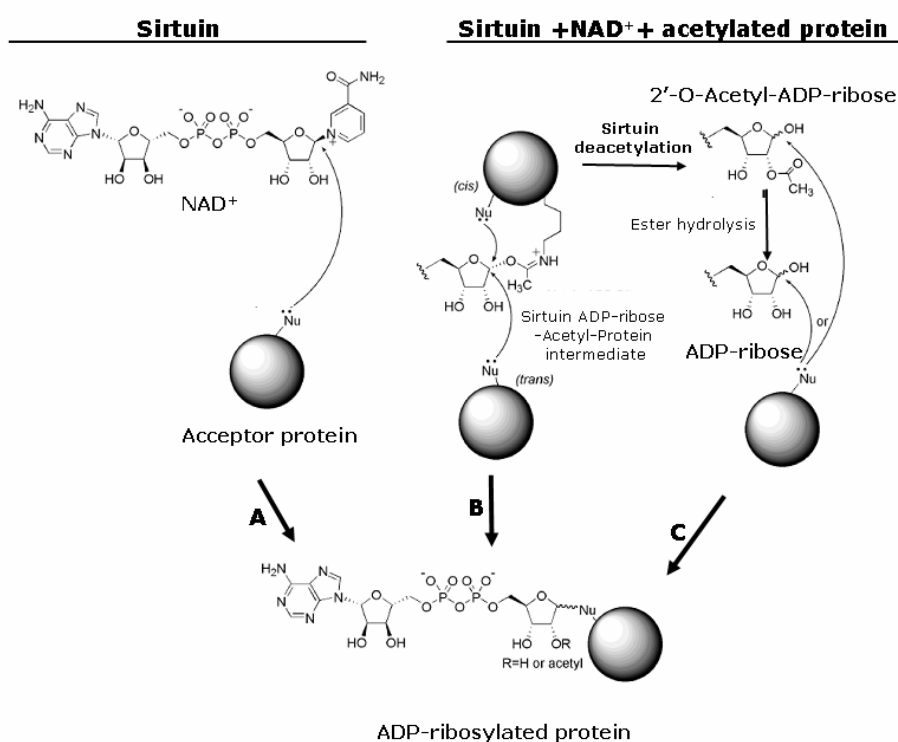


Figure 13. Sirtuins mediated ADP-ribosylation: proposed pathways. An acetylated substrate is not required and the transference of ADP-ribose moiety occurs directly from NAD⁺ (A). This pathway requires an acetylated peptide and NAD⁺ to generate an O-alkylamidate intermediate which subsequently reacts with a nucleophile (B). The OAADPr generated in a sirtuin catalyzed deacetylation reaction, subsequently reacts with the protein substrate (C) (Adapted from Denu et al., 2005).

4 The diversity of the Sirtuin family

Sirtuins are found in a variety of organisms ranging from bacteria to humans, indicating a high degree of conservation throughout evolution (Brachmann et al., 1995). Bacterial genomes usually encode only one sirtuin while eukaryotes usually have multiple Sirtuins (Sauve et al., 2006). Phylogenetic analysis revealed the presence of a conserved sequence of ~250aa core domain in Sirtuins (Frye, 1999; Frye, 2000). Moreover, some Sirtuins, including the yeast SIR2, present significant extensions that flank the conserved core domain at the N and C termini (Sauve et al., 2006). Indeed, there are some evidences suggesting the involvement of these extensions in the substrate-specific recognition and subcellular localization (Cuperus et al., 2000). In the case of the yeast SIR2, these extensions contain binding sites for SIR4, which forms a complex with SIR2 in the cell (Moazed, 1997). The first evidence that the SIR2 proteins could be involved in functions other than transcriptional silencing came from the presence of the *Leishmania major* SIR2 related protein in cytoplasmic granules (Zemzoumi et al., 1998). Sirtuins have been found in a variety of subcellular localizations. Concerning human enzymes, SIRT1, SIRT6 and SIRT7 are localized in the nucleus; SIRT2 is localized in the cytoplasm; while SIRT3, SIRT4 and SIRT5 presented a mitochondrial localization (Figure 14) (for review, Michan and Sinclair, 2007).

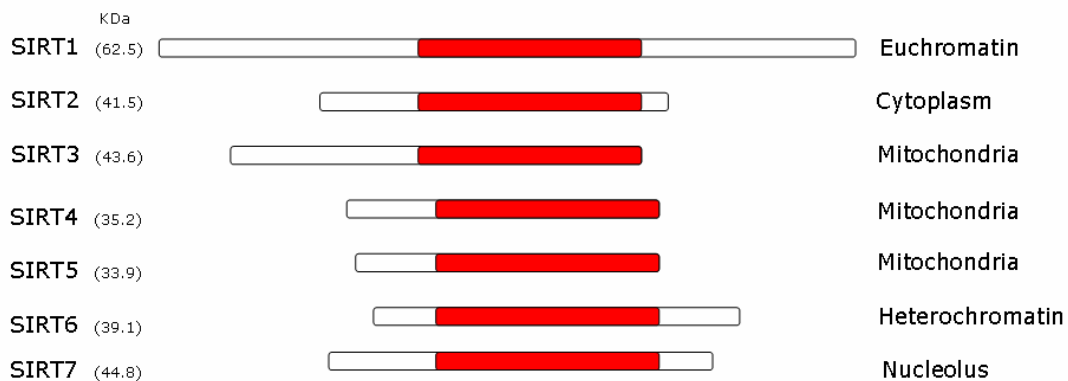


Figure 14. Mammalian Sirtuins. Schematic representation of the seven human Sirtuins, which present core domain conservation and different subcellular localizations. N and/or C terminal extensions of different length may flank the core domain (Adapted from Frye et al., 2000).

4.1 Sirtuin structural properties

Crystallography studies of archaeal Sirtuins, like SIR2 from *Archaeoglobus fulgidus* 1 (SIR2Af1) (Bell et al., 2002) and SIR2Af2 (Avalos et al., 2002; Avalos et al., 2004; Avalos et al., 2005), human SIRT2 (Finnin et al., 2001), yeast Hst2 (Zhao et al., 2003; Zhao et al., 2004), bacterial CobB (Zhao et al., 2004) and SIR2 homologue from *Thermatoga maritima* (SIR2Tm) (Avalos et al., 2005) have provided significant information on the structure of the Sirtuins' catalytic conserved core domain and its binding to ligands and co-factors. Indeed, the catalytic core of Sirtuins consists of two characteristic domains (Figure 15) (Min et al., 2001; Bell et al., 2002; Avalos et al., 2002; Avalos et al., 2004; Avalos et al., 2005; Finnin et al., 2001; Zhao et al., 2003; Zhao et al., 2004). The larger domain is a variant of the classical Rossmann fold, which is commonly found in proteins that bind NAD⁺/NADH or NADP/NADPH (Rossmann et al., 1978). The small domain is comprised by a zinc binding domain and a flexible loop. The position of the small domain in relation to the large domain varies depending on different Sirtuins structures and is influenced by ligand binding as well as contact with other proteins (Sauve et al., 2006). The NAD⁺ molecule and the acetylated peptide bind to the enzyme in the large groove between the interfaces of the two domains.

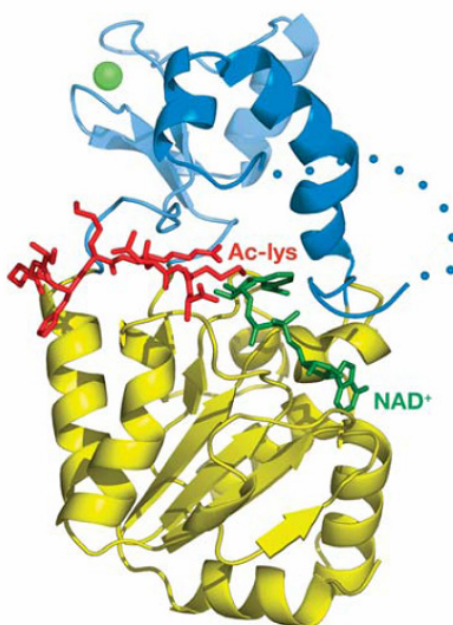


Figure 15. Structure of a Sirtuin bound to acetylated peptide and NAD⁺. The catalytic core domain of Sirtuins is composed by a large and a small domain. The large domain is composed by a classical Rossmann fold and is coloured yellow. The small domain is coloured blue and is composed of a zinc binding domain (light blue) and of a flexible loop (royal blue). The NAD⁺ molecule (green) and the

acetylated peptide (red) bind at the interface between the two domains (Adapted from Sauve et al., 2006).

5 The biological role of Sirtuins

5.1 Sirtuins, caloric restriction and aging

Several studies have been suggesting the involvement of Sirtuins in the promotion of longevity, particularly the one associated with caloric restriction regimens. Indeed, the discovery that extra copies of Sirtuins promote longevity in *C. elegans*, and the correlation of yeast longevity and Sirtuins' activity, were two key early findings (Tissenbauman and Guarente, 2001; Kaeberlein et al., 2004).

The accumulation of extrachromosomal rDNA circles, which results from homologous recombination between rDNA repeats, has been described as a major cause of aging in *S. cerevisiae* (Sinclair and Guarente, 1997). Indeed, the introduction of a single extrachromosomal rDNA circle into a young cell caused premature aging. Moreover, increased instability of rDNA and reduced longevity was observed by mutation of the SGS1 gene, a homologue of the premature aging disease gene, WRN (Werner syndrome) (Sinclair et al., 1997). The introduction of an extra copy of SIR2 gene increased the replicative lifespan in yeast while the deletion of SIR2 increased the accumulation of extrachromosomal rDNA circles shortening lifespan (Kaeberlein et al., 1999). Moreover, the short lifespan of a SIR2 mutant was suppressed by the replication fork gene (FOB1), which reduces rDNA recombination and extrachromosomal rDNA circle formation.

A role for sirtuins mediating caloric restriction and displaying an increased replicative lifespan was for the first time attributed in *S. cerevisiae* (Lin et al., 2000; Lamming et al., 2005). A recent report shows that the NAD⁺-precursor nicotinamide riboside elevates NAD⁺ levels, increases Sir2 function, and prolongs life span in yeast (Belenky et al., 2007).

The metazoan worm, *Caenorhabditis elegans*, also displayed lifespan extension when carrying extra copies or expression of SIR2 gene by a mechanism that depends on the forkhead transcriptional factor DAF-16 (abnormal dauer formation 16), the downstream target of the insulin/IGF (insulin like growth factor)-1 signalling pathway (Tissenbaum et al., 2001). Indeed SIR-2.1, the metazoan homologue of the yeast SIR2, was shown to associate with 14-3-3 proteins, which direct its interaction with DAF-16 in an insulin/IGF-1-independent manner under stress conditions (Wang et al., 2006).

The overexpression of SIR2 gene in *Drosophila melanogaster* also induced increased lifespan through a pathway related with caloric restriction (Rogina et al., 2004).

5.2 Mammalian Sirtuins

A number of substrates have been identified in the several Sirtuins that suggest their involvement in controlling different biological processes. However, these substrates could generally be divided into three major groups: (i) chromatin structure and transcription, (ii) apoptosis regulation and (iii) metabolic regulation (Sauve et al., 2006).

(i) Chromatin structure and transcription

The mammalian SIRT1, as the yeast SIR2, also has a role in heterochromatin formation through the deacetylation of histones' lysine residues at positions 9 and 26 in histone H1, 14 in H3 and 16 in H4 (Imai et al., 2000; Vaquero et al., 2004). Moreover, a number of transcription factors have been identified as SIRT1 substrates. Among them are the: TAF_I68 [TBP(TATA-box-binding protein)-associated factor I 68], which regulates transcription of RNA polymerase I (RNA Pol I) and its deacetylation by SIRT1 represses the transcription of RNA Pol I *in vitro* (Muth et al., 2001); the acetyltransferases PCAF [p300/cAMP-response-element binding protein-associated factor] and GCN5 interact with SIRT1 and mediate the formation of a complex with the muscle-specific transcription factor MyoD (myogenic differentiation). When this complex is recruited to chromatin, SIRT1 deacetylates its proteins and also Lys9 and Lys14 of H3 in the myogenin and myosin heavy chain promoters. These events lead to the inhibition of muscle gene expression, which produces retardation of muscle differentiation (Fulco et al., 2003); The MEF2 (MADS box transcription enhancer factor 2) family of transcription factors involved in muscle differentiation is also regulated by SIRT1 (Zhao et al., 2005).

Interestingly, SIRT7, a nucleolar sirtuin, activates RNA Pol I mediated transcription and expression of ribosomal genes. Indeed, SIRT7 was found to be part of the RNA pol I machinery, interacting not only with the promoter but also with transcribed regions of rDNA locus and histones H2a and H2b (Ford et al., 2006).

(ii) Apoptosis regulation

The tumour suppressor factor p53 has been described as a substrate deacetylated by the mammalian (human and mouse) SIRT1. Indeed, SIRT1 has been shown to

deacetylate multiple lysine residues of p53 (Luo et al., 2001; Cheng et al., 2003). The p53 deacetylation mediated by SIRT1 led to the inhibition of its transactivation activity and suppresses apoptosis induced by oxidative stress and DNA damage (Cheng et al., 2003; Vaziri et al., 2001). Furthermore, thymocytes from SIRT1 deficient mice, revealed p53 hyperacetylation after DNA damage and increased sensitivity to ionizing radiation (Cheng et al., 2003). However, recent reports suggest that despite p53 deacetylation by SIRT1, the regulation of this pathway might involve other complex mechanisms in addition to p53 deacetylation. Indeed, the SIRT1 protein had little effect on p53-dependent transcription of transfected or endogenous genes, and did not affect the sensitivity of thymocytes and splenocytes to radiation-induced apoptosis, suggesting that SirT1 does not affect many of the p53-mediated biological activities (Kamel et al., 2006). Furthermore, the inhibition of SIRT1 catalytic activity increases p53 acetylation but does not alter cell survival following DNA damage in different cells (Solomon et al., 2006). Similar to p53, the deacetylation of the tumour suppressor p73 by SIRT1 suppresses its transcriptional activity and inhibits apoptosis in HEK-293 cells (Dai et al., 2007).

Another pathway through which SIRT1 promotes cell survival involves the deacetylation of a DNA-repair factor and inhibitor of Bax-mediated apoptosis, Ku70. Indeed, once deacetylated, Ku70 complexes with the proapoptotic factor Bax sequestering it away from the mitochondria and thus preventing it from triggering apoptosis in HEK-293 cells (Cohen et al., 2004). Moreover, SIRT1 interacts with the cell cycle and apoptosis regulator, E2F1, affecting the cell's sensitivity to DNA damage (Wang et al., 2006).

The Forkhead box class O (FOXO) transcription factors represent another SIRT1-regulated pathway to promote cell survival. Indeed, three out of four FOXO transcription factors are deacetylated by SIRT1. Moreover, SIRT1 also affects Foxo3a function in mammalian cells, including neurons and fibroblasts, reducing apoptosis in response to stress stimuli, but increasing the expression of DNA repair and cell-cycle checkpoint genes (Motta et al., 2004; Brunet et al., 2004). Increased acetylation of FOXO4, induced by hydrogen peroxide treatment, suppresses its transcriptional activation, and is reverted by the interaction with SIRT1, which enhances the cell defences against oxidative stress through the expression of the growth arrest and DNA repair protein, GADD45 (growth arrest and DNA-damage-inducible α) (van der Horst et al., 2004; Kobayashi et al., 2005).

Furthermore, SIRT1 can also deacetylate NF-kB, repressing target gene expression (Yeung et al., 2004). NF-kB is maintained in inactive form in the cytoplasm and translocates to the nucleus in response to stress, inflammation and infection. Once

in the nucleus, NF- κ B induces the transcription of cell-cycle, proliferation, migration and apoptosis genes (Piva et al., 2006).

A role of SIRT6 in the DNA repair has been suggested due to the impairment of base excision repair observed in SIRT6 knockout mice (Mostoslavsky et al., 2006).

(iii) Metabolic regulation

Do mammalian Sirtuins extend life span by modulating metabolism?

Even though the involvement of mammalian Sirtuins in the caloric restriction (CR) response and regulation of metabolism has been intensively studied, much more experimental work is required before such an involvement can be fully evaluated.

Relevant functions in metabolism regulation have been attributed to SIRT1, SIRT3 and SIRT4. SIRT1 is involved in fat mobilization in white adipose tissue through its binding and repression of genes involved in adipogenesis, such as PPAR- γ (peroxisome proliferator-activated receptor γ) and aP2 (fatty acid binding protein) (Picard et al., 2004). Furthermore, SIRT1 regulates adiponectin, which is an adipocyte-derived hormone, whose plasmatic levels correlate inversely with adiposity (Qiao et al., 2006). Moreover, SIRT1 represses glycolysis and increases hepatic glucose output by deacetylating PGC-1 α (PPAR- γ co-activator 1 α) (Rodgers et al., 2005). Recent observations indicate that SIRT2, a predominantly cytoplasmic sirtuin with specificity for acetylated α -tubulin (North et al., 2003), also regulates the differentiation of adipocytes (Jing et al., 2007). Indeed, SIRT2 directly interacts with FOXO1, mediates its deacetylation, and inhibits adipocyte differentiation. In contrast, SIRT2 knockdown is associated with hyperacetylation, enhanced phosphorylation, cytosolic localization of FOXO1, and enhanced adipogenesis (Jing et al., 2007).

When overexpressed in pancreatic β -cells, SIRT1 led to enhanced glucose-stimulated insulin secretion and ATP production in mice (Moynihan et al., 2005; Bordone et al., 2006). The positive regulation of insulin secretion by the pancreatic β cells occurs by SIRT1-mediated repression of the uncoupling protein (UCP) gene UCP2 by binding directly to the UCP2 promoter (Bordone et al., 2006).

The mitochondrial SIRT3 plays a role in adaptive thermogenesis in brown adipose tissue and activates mitochondrial function. Indeed, sustained expression of SIRT3 decreases mitochondrial membrane potential and reactive oxygen species production, while increasing cellular respiration (Shi et al., 2005).

Moreover, Sirtuins can also directly control enzymes involved in metabolism such as Acetyl-CoA synthetases (AceCSs) (Hallows et al., 2006; Schwer et al., 2006). In mammals, Acetyl-CoA synthetase 1 (AceCS1) and Acetyl-CoA synthetase 2

(AceCS2) give origin in cytoplasm and mitochondria, respectively, to Acetyl-CoA (AceCoA) from the acetate of the diet or from endogenous reactions. The AceCoA is central to the synthesis of fatty acids, amino acids, ketone bodies and to the tricarboxylic acid cycle. Therefore, SIRT1 and SIRT3 deacetylate and activate AceCS1 and AceCS2, respectively (Hallows et al., 2006; Schwer et al., 2006). Since during CR the expression of SIRT1 and SIRT3 is induced, it seems plausible that these Sirtuins regulate how much carbon shuttles to the tricarboxylic acid cycle for ATP production (Michan and Sinclair, 2007). The mitochondrial sirtuin, SIRT4, regulates aminoacid stimulated insulin secretion by the pancreatic β cells (Haigis et al., 2006). The glutamate dehydrogenase (GDH) regulates glutamate and glutamine metabolism, promotes ATP synthesis and enhances insulin secretion. The ADP-ribosylation of GDH by SIRT4 leads to GDH inhibition, while an increase in GDH activity and consequently higher insulin levels, up-regulation of aminoacid-stimulated insulin secretion and secretion of insulin in response to glutamine has been observed in pancreatic islets of SIRT4 knockout mice (Haigis et al., 2006). A similar phenotype is observed in pancreatic islets of mice under CR, suggesting that SIRT4 is down-regulated by CR, thus inducing the expression of GDH and allowing glutamine to serve as an insulin secretagogue.

5.3 The parasite SIR2 homologues

5.3.1 *Leishmania* spp.

The screening of a *Leishmania* cDNA library using an anti-*L. major* glutathione binding protein immune serum led to the identification of a SIR2-like gene in *L. major* (Yahiaoui et al., 1996). This cDNA sequence encodes a *Leishmania* protein named LmSIR2RP1, with approximately 41% homology with the yeast SIR2. Moreover, immunological approaches revealed the presence of LmSIR2RP1 homologues in others *Leishmania* species. In 2002, Vergnes and colleagues cloned and sequenced a gene encoding an homologous SIR2 protein from *L. infantum* (LiSIR2RP1) that reveals a 93% identity with that of *L. major* SIR2 (Vergnes et al., 2002). Additionally, two other related sequences (LmSIR2RP2 and LmSIR2RP3) can be found in the *Leishmania* genome database [*L. major* sirtuin (CAB55543) and *L. major* cobB (LmjF34.2140), respectively], which await further investigations. Interestingly, LmSIR2RP1 was the first member of the SIR2 family to exhibit a cytosolic localization, with no reactivity in the nucleus detected by either immunofluorescence or Western Blot analysis. Even though this protein is detected in all *Leishmania* developmental stages, it has a heterogeneous cytosolic distribution, in granules of different size and numbers, depending on the life stage

and *Leishmania* specie (Zemzoumi et al., 1998). Moreover, this protein is among the excreted and secreted *L. major* antigens, since it was detected by immunoprecipitation in the supernatant of a metabolically labelled culture (Zemzoumi et al., 1998). Indeed, antibodies against LmSIR2RP1 have been detected in the sera of dogs naturally infected by *Leishmania*, suggesting the interaction of this parasite protein with host cells (Cordeiro-da-Silva et al., 2003). When constitutively expressed in mouse L929 fibrosarcoma cells, LmSIR2RP1 induced in the host cells a senescence phenotype that rendered the host cells more permissive to *Leishmania* infection (Serenio et al., 2005). Moreover, LmSIR2RP1 triggers specific murine B cell effector functions, inducing the production of parasite-specific antibodies which were lytic in the presence of complement, and restrains the capacity of the parasites to infect macrophages (Silvestre et al., 2006).

The overexpression of LiSIR2RP1 or LmSIR2RP1 protein led to increased survival of the *L. infantum* amastigotes, which displayed resistance to apoptosis-like death (Vergnes et al., 2002). Interestingly, no similar effect was observed in the survival of promastigote forms under similar culture conditions. This suggested that the protein, alone or in combination with other cellular factors, may participate in the control of cell death in these pathogenic organisms. Immunoprecipitation experiments conducted with the aim of identifying SIR2RP1 putative substrates or partners, revealed the existence of an interaction between SIR2RP1 and the heat shock protein 83 (HSP83) in *Leishmania* (Adriano et al., 2007). However, the level of SIR2RP1 expression does not influence the level of HSP83 acetylation.

5.3.2 *Trypanosoma* spp

African trypanosomes, e.g. *Trypanosoma brucei*, grow in mammalian vasculature and are the agents of sleeping sickness. Once in the bloodstream, they evade the host's immune response by continually changing their variant surface glycoprotein (VSG) (reviewed in Cross et al., 1998). VSG variation occurs either by gene conversion or by the transcriptional activation of a new expression site. Similarly to *Leishmania*, *T. brucei* also has genes encoding three SIR2 protein homologues (TbSIR2rp1-3) while *T. cruzi* has only genes encoding the SIR2 homologue proteins 1 and 3 (Ivens et al., 2005; Alsford et al., 2007). In the search for factors that influence the chromatin structure and gene expression, Garcia-Salcedo and colleagues reported in 2003 that a SIR2 homologue in *T. brucei*, termed TbSIR2RP1, is a chromosome-associated protein that catalyses the NAD⁺-dependent ADP-ribosylation and deacetylation of histones. Moreover, TbSIR2RP1

appears to have a role in the mechanism of DNA repair, probably by converting target regions of chromatin into a more relaxed or open state (Garcia-Salcedo et al., 2003; Alsford et al., 2007). Even though TbSIR2RP1 protein is involved in telomeric silencing in the bloodstream stage, it is not required for VSG gene silencing or antigenic variation. Two other SIR2 homologues, namely TbSIR2RP2 and TbSIR2RP3, were shown to be localized in the mitochondria of the *T. brucei* bloodstream-stage (Alsford et al., 2007).

5.3.3 *Plasmodium* spp

Plasmodium falciparum is responsible for the most severe form of malaria in humans. A sirtuin homologue of *P. falciparum*, termed PfSIR2, was shown to be involved in the epigenetic transcription control of a large number of sub-telomeric genes that are vital for virulence (Duraisingh et al., 2005). These include the multigene families var and rifin, both encoding variantly-expressed antigens exposed on the surface of infected erythrocytes during a blood-stage malarial infection (Craig and Scherf, 2001). Indeed, the disruption of PfSIR2 led to the abolishment of the mutually exclusive expression of var genes and upregulation of multiple var gene expression (Duraisingh et al., 2005). A further study revealed that H4 was more highly acetylated within an active sub-telomeric var gene than within a silent one, and that this hyperacetylation was mutually exclusive with the presence of PfSir2 (Freitas-Junior et al., 2005). Indeed, these studies revealed that the heterochromatin silencing mediated by PfSIR2 could control the expression of var genes. Furthermore, the PfSIR2 protein exhibits both NAD⁺-dependent deacetylase and ADP-ribosyltransferase of histones (Merrick et al., 2007; Chakrabarty et al., 2008).

6 Modulation of Sirtuins enzymatic activity

6.1 Activators

The first molecules identified as SIRT1 activators were plant polyphenolic metabolites belonging to three structural classes: chalcones (buteine, Figure 16, A), flavones (quercetin, Figure 16, B) and stilbenes (resveratrol, Figure 16, C) (Howitz et al., 2003). However, resveratrol has revealed to be the most potent activator, given its ability to activate the enzyme *in vitro* and also to extend the *S. cerevisiae* lifespan in a SIR2 dependent manner (Howitz et al., 2003). Structure-activity studies conducted towards SIRT1 activation have identified the hydroxyl groups at

positions 3 and 5 of the A ring, as crucial for the enzyme activation (Figure 16, C) (Yang et al., 2007). Furthermore, imidazoquinoxaline and pyrroloquinoxaline (Figure 16,D) were identified *in vitro* as SIRT1 activators (Nayagam et al., 2006). More recently, novel small-molecule activators of SIRT1 [SRT2183 (Figure 16, E), SRT1460 (Figure 16, F), and SRT1720 (Figure 16, G)] have been described (Milne et al., 2007). Although structurally unrelated with any of the previous SIRT1 activators, these compounds revealed to be more potent, orally bioavailable and active *in vivo* (Milne et al., 2007).

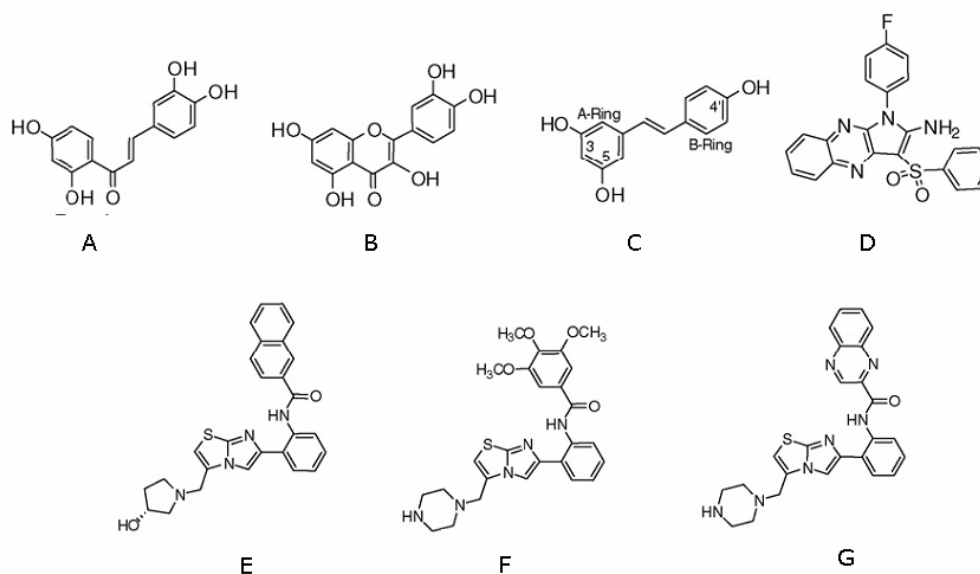


Figure 16. Examples of sirtuin activators. Buteine (A), Quercetin (B), Resveratrol (C), Pyrroloquinoxaline (D), SRT2183 (E), SRT1460 (F) and SRT1720 (G).

6.2 Inhibitors

While the class I and II histone deacetylases had already been validated as anticancer drug targets with some of the inhibitors having reached clinical trials, less is known about the inhibition of the class III members (Johnstone et al., 2002). However, recent findings have suggested a direct link between the activity of Sirtuins and diseases such as cancer, HIV and Parkinson (Pagans et al., 2005; Vaziri et al., 2001; Bereshchenko et al., 2002; Ota et al., 2006; Outeiro et al., 2007). The first reports on Sirtuin inhibitors identified, besides the physiological inhibitor nicotinamide (Figure 17Figure 17Figure 17Figure 17Figure 17), sirtinol (Figure 17, B) and splitomicin (Figure 17, C) (Grozier, CM et al., 2001; Bedalov, A. et al., 2001). Since they all exhibited weak inhibitory properties, subsequent structure-activity studies have led to the identification of more potent derivatives

such as HR73 and β -phenylsplitomicin (Pagans et al., 2005; Neugebauer et al., 2008). Furthermore, a high-throughput screening against the human sirtuin SIRT1 has led to the identification of indoles (Figure 17, D) as the most potent ($IC_{50} < 0.1 \mu M$) and selective inhibitors described to date (Napper et al., 2005). Kinetic analysis revealed mixed-type inhibition of deacetylation, as well as inhibition of nicotinamide- NAD^+ exchange reaction. In addition, drugs that mimic adenosine (suramin, Figure 17, E) or target enzymes or receptors, like kinases, that bind to adenosine-containing cofactors or ligands were identified as human SIR2 inhibitors (Howitz, KT et al., 2003; Trapp, J et al., 2006). Most notable was a bis(indolyl)maleimide which inhibited SIRT1 and SIRT2 with IC_{50} values of 3.5 and $0.8 \mu M$, respectively. Competition assays with NAD^+ and modelling studies have indicated that these bis(indolyl)maleimides probably bind to the adenosine pocket. Suramin was initially used to treat sleeping sickness and onchocerciasis. Moreover, in a recent study, the crystal structure of SIRT5 bound to suramin revealed that the two halves of the symmetrical suramin molecule-induced SIRT5 dimerization mediated primarily through suramin itself (Schuetz et al., 2007). Indeed, suramin binds within the active site and partially fills pockets where the nicotinamide-ribose moiety of NAD^+ binds in the enzyme-substrate complex, as well as portions of the adjacent acetylated-peptide binding site. The Oxadiazole-carbonylaminothiurea derivatives were also reported as a new class of SIRT1 and SIRT2 inhibitors (Huhtiniemi et al., 2006 and 2008)

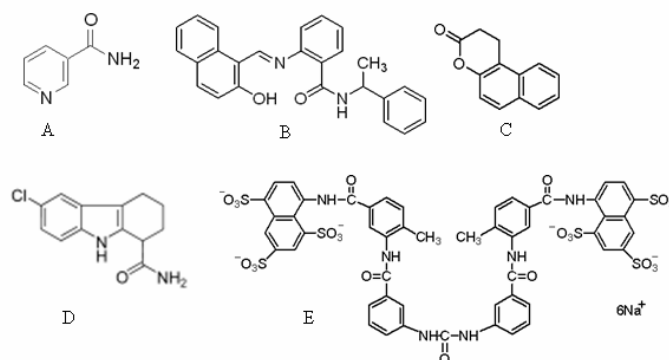


Figure 17. Examples of sirtuin inhibitors. Nicotinamide (A), Sirtinol (B), Splitomicin (C), an Indole derivative, 6-chloro-2,3,4,9-tetrahydro-1-H-carbazole-1-carboxamide (D) and Suramin (E).

Several acetyl-lysine analogues have been used to explore mechanistic differences among protein deacetylases (Fatkins et al., 2006; Smith and Denu, 2007). Thioacetyl-lysine and trifluoro-acetyl lysine containing peptides have been identified as potent inhibitors of Sirtuins' deacetylases, but they are mechanistically distinct

(Smith and Denu, 2007). Indeed, while the trifluoro-acetyl lysine peptide displays enzyme inhibition by competition with the substrate, the thioacetyl-lysine peptide stalls the enzymatic reaction at an intermediate after nicotinamide formation (Smith and Denu, 2007).

6.3 Endogenous regulators

There is some evidence that the cellular levels of the product inhibitor, nicotinamide, and the cofactor NAD^+ might influence Sirtuins' activity (Araki et al., 2004; Sauve et al., 2005). Indeed, CD38, which is localized in the inner nuclear membrane, hydrolyses NAD^+ to nicotinamide (Askoy et al., 2006). In a $\text{CD38}^{-/-}$ mice, NAD^+ levels are increased and the SIRT1 substrate p53 is less acetylated (Askoy et al., 2006). Moreover, nicotinamide phosphoribosyltransferase (Nampt), is induced after some forms of stress, and converts nicotinamide to nicotinamide mononucleotide, which then reacts with ATP to regenerate NAD (Yang and Sauve, 2006). The overexpression of Nampt increases NAD levels and induces SIRT1 activity, with changes in gene expression similar to those in cells overexpressing SIRT1 (Revollo et al., 2004).

The poly(ADPribose) polymerase (PARP) is another enzyme that uses NAD^+ . Localized in the nucleus, this protein is involved in DNA repair and cleaves NAD^+ into nicotinamide and ADP-ribose (Zhang, 2003). The activation of PARP after DNA damage quickly depletes cells of NAD^+ with an increase in nicotinamide levels, which may suppress the activity of the Sirtuins (Zhang, 2003).

Nicotinamide, as a precursor of the NAD^+ , inhibits Sirtuins by blocking the regeneration of NAD^+ through the interception of an ADP-ribosylenzyme-acetyl peptide intermediate (Jackson et al., 2003; Porcu and Chiarugi, 2005).

Two recent reports have suggested that SIRT1 sumoylation (C- terminal) or binding of active regulator of SIRT1 (AROS) (N-terminal) can activate SIRT1 (Kim et al., 2007; Yang et al., 2007). However, the mechanistic basis for increased SIRT1 activity upon sumoylation or AROS binding remains to be determined.

Chapter V

Objectives and results

1 Scope of the thesis

The necessity to discover new molecules with antileishmanial activity is explicit. Among the several strategies available, the most fruitful has been the discovery of new applications for existing drugs. The recent introduction of the anticancer drug miltefosine as an effective oral antileishmanial drug, led us to explore the potential of cisplatin (another anticancer molecule) against *L. infantum*. At the same time, a more rational approach was conducted, aiming at the identification of new *Leishmania* virulence factors. By the time this research project was initiated, the proteins belonging to the SIR2 family were already known to be involved in the regulation of several functions in eukaryotic cells, including transcriptional repression, recombination, cell cycle, cellular responses to DNA-damaging agents, microtubule organization and longevity (reviewed in North and Verdin, 2004). Moreover, functions as NAD⁺-dependent deacetylases and ADP-ribosyltransferases of different cellular substrates, including histones, were already attributed to some members of this family (reviewed in North and Verdin, 2004). Three SIR2 homologues were identified in the *Leishmania* genome (Yahiaoui et al., 1996; Ivens et al., 2005; Peacock et al., 2007). One of them, named LmSIR2, was discovered to be localized in the cytosol (Zemzoumi et al., 1998). Furthermore, indirect molecular and biological approaches demonstrated the potential role of LmSIR2 and LiSIR2 genes in parasite survival, due to an inherent resistance to apoptosis-like death (Vergnes et al., 2002). Accordingly, we decided to investigate the role of *L. infantum* cytosolic SIR2 protein in the parasite infectious cycle. The essentiality of this protein in the parasite's survival and virulence made LiSIR2 protein an attractive drug target. Subsequent studies were therefore performed in order to characterize its enzymatic function and search for specific inhibitors with antileishmanial activity.

The experimental results obtained in this thesis are organized in 8 chapters. **Chapter 2** describes the antileishmanial activity of cisplatin against *L. infantum* promastigotes and amastigotes. In **Chapter 3**, a "reverse" genetic approach is used to disclose the biological function of the cytosolic SIR2 in *Leishmania*'s survival and virulence. **Chapter 4** describes an *in silico* approach to find *Leishmania* SIR2 inhibitors based on minor differences in ligand binding and catalytic domain, as compared to its human counterpart. Furthermore, the enzymatic functions of the

LiSIR2-related protein 1 are described and at least one substrate identified in **Chapter 5**. The *in vitro* antileishmanial activity of bisnaphthalimidopropyl (BNIP) derivatives is reported in **Chapter 6** and **7**, while in **chapter 8** their inhibitory property toward the NAD⁺-dependent deacetylase activity of LiSIR2RP1 is described. In **Chapter 9**, the potential of BNIP derivatives to treat VL is evaluated using a murine Balb/c model, chronically infected with *L. infantum*.

2 Results

2.1 Characterization of the anti-*Leishmania* effect induced by cisplatin, an anticancer drug.

The cis-diamminedichloroplatinum(II), known as cis-DDP or cisplatin is a widely used drug in cancer chemotherapy. Although a recent study has shown the anti-*Leishmania* activity of some cis-DDP derivatives, the cytotoxic properties were measured only on promastigotes, the insect vector form of the parasite. In this study the effect of cis-DDP on promastigotes and amastigotes, the vertebrate stage of the parasite is reported. The IC_{50} , determined by flow cytometry, after 72 h of drug incubation was four times higher, $7.73 \pm 1.03 \mu M$ in the case of promastigotes compared to axenic amastigotes, $1.88 \pm 0.10 \mu M$. In intracellular amastigotes the IC_{50} , determined by counting the parasite index was $1.85 \pm 0.22 \mu M$. By using flow cytometry, two patterns of cell cycle changes was observed: cis-DDP treated promastigotes and amastigotes accumulated in S phase and G2 phase, respectively. The cis-DDP response was also found to involve an "apoptosis-like" death of both promastigotes and amastigotes. However, DNA fragmentation was only detected in promastigote forms. In contrast mitochondrial transmembrane potential loss was observed for both stages of the parasite. Upon incubation of parasites with the drug an increase on GSH and GSSG levels and reactive oxygen species could be detected in the case of promastigote. Moreover, a slight increase of GSH level was detected on amastigote form. Taken together, these observations indicate that amastigotes are more sensitive to cis-DDP when compared to promastigotes. However, the signalling pathways leading to cell death could be different.

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Characterization of the anti-*Leishmania* effect induced by cisplatin, an anticancer drug

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Abstract

The *cis*-diamminedichloroplatinum(II), known as *cis*-DDP or cisplatin is a widely used drug in cancer chemotherapy. Although a recent study has shown the anti-*Leishmania* activity of some *cis*-DDP derivatives, the cytotoxic properties were measured only on promastigotes, the insect vector form of the parasite. In this study the effect of *cis*-DDP on promastigotes and amastigotes, the vertebrate stage of the parasite is reported. The IC₅₀, determined by flow cytometry, after 72 h of drug incubation was four times higher, $7.73 \pm 1.03 \mu\text{M}$ in the case of promastigotes compared to axenic amastigotes, $1.88 \pm 0.10 \mu\text{M}$. In intracellular amastigotes the IC₅₀, determined by counting the parasite index was $1.85 \pm 0.22 \mu\text{M}$. By using flow cytometry, two patterns of cell cycle changes was observed: *cis*-DDP treated promastigotes and amastigotes accumulated in S phase and G2 phase, respectively. The *cis*-DDP response was also found to involve an “apoptosis-like” death of both promastigotes and amastigotes. However, DNA fragmentation was only detected in promastigote forms. In contrast mitochondrial transmembrane potential loss was observed for both stages of the parasite. Upon incubation of parasites with the drug an increase on GSH and GSSG levels and reactive oxygen species could be detected in the case of promastigote. Moreover, a slight increase of GSH level was detected on amastigote form. Taken together, these observations indicate that amastigotes are more sensitive to *cis*-DDP when compared to promastigotes. However, the signaling pathways leading to cell death could be different.

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Keywords: Cisplatin; *Leishmania infantum*; Anticancer drug; Cell death

1. Introduction

Cis-DDP is a widely used drug in treatment of several types of tumors including testicular and ovarian cancers (Fuentes et al., 2002). This chemotherapeutic

agent is generally recognized as a DNA-damaging drug. Although in responsive cells, it induces apoptosis, which typically involves cytochrome *c* release from mitochondria and the subsequent activation of caspase-9 and 3. The signaling pathways connecting drug-induced DNA modifications to the execution phase are still under investigation (Mandic et al., 2002). One of us has recently examined the effects of *cis*-DDP on various human pancreatic cell lines carrying a murine α 1,3-galactosyltransferase encoding gene, allowing the cells

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to express the Gal α 1,3Gal xenoantigen *in vitro* and *in vivo* when implanted into nude mice (Ouaissi, 2004). The results showed that the expression of the α -Gal xenoantigen could induce an increased susceptibility of human pancreatic cancer cells to *cis*-DDP treatment. Biochemical investigations suggested that the drug induced cell death by necrosis rather than apoptosis (Ouaissi, 2004).

Leishmaniasis is caused by the protozoan parasite *Leishmania* sp., which is transmitted to the vertebrate-host by the bite of the phlebotomine sandfly. The different pathological manifestations vary with the species which are widely distributed in tropical and subtropical areas and also are commonly found in the Mediterranean basin. The extent and severity of this group of diseases is an important health problem (Berman, 1997). *Leishmania* parasite has a complex life cycle that includes different morphological forms. Within the insect vector, the parasites replicate as non-infective promastigotes which transform into infective metacyclic promastigotes. In the mammalian host, the infective promastigotes invade the macrophages and differentiate into amastigotes which are the proliferative forms within the vertebrate hosts.

The chemotherapy of parasitic diseases is limited to the use of pentavalent antimonials such as sodium stibogluconate (Pentostan), *N*-methylglucamine (Glucantime), amphotericin B or pentamidine (Murry, 2001). These drugs are toxic and difficult to administrate because of their long term treatment and high cost (Croft, 1986; Oullette and Papadopolou, 1993; Modabber, 1995). In addition, even in successful treated patients the presence of the parasite has been reported using polymerase chain reaction with the parasite specific oligonucleotide probes (Guevara et al., 1993). Therefore, investigations are still in progress to discover new antileishmanial compounds.

It is well known that many antiprotozoal drugs bind to the DNA (Croft and Coombs, 2003). Investigations conducted on kinetoplastid parasites (*Trypanosoma cruzi* and *Leishmania*) using *cis*-DDP and its analogs have shown that some of these compounds could exert cytotoxic activity (Osuna et al., 1987; Nguewa et al., 2005), suggesting therefore the existence of some parasitic and cancer cell shared regulatory pathways leading to the *cis*-DDP complexes induced cell death. Given that these studies were mainly done with the insect stage of the parasite, we thought it would be interesting to evaluate the effect of the drug on both promastigote and amastigote stages and examine the physiological alterations and the type of drug-induced cell death.

In the present report, we showed that *cis*-DDP induced alteration of the cell cycle in both promastigote

and amastigote forms with the latter being highly sensitive to the drug induced cell death. Moreover, mitochondrial membrane depolarization could be demonstrated in both stages of the parasite. However DNA degradation, presence of reactive oxygen species and a significant increase in GSH and GSSG levels could only be detected in the case of *cis*-DDP treated promastigotes, suggesting that the pathways leading to parasite death might be different.

2. Materials and methods

2.1. *Cis*-DDP

The *cis*-DDP compound was purchased from Merck (Saint Romain, Lyon, France). A commercial stock solution of 1 mg/ml (batch no. 3019) was stored at room temperature. Working solutions were freshly prepared using culture medium till the desired final concentration for the different assays.

2.2. Parasites and growth inhibition assays

Leishmania infantum (clone MHOM/MA671TMAP-263) promastigotes were grown at 27 °C in RPMI medium (Gibco) supplemented with 10% of heat inactivated fetal bovine serum (FBS-Gibco), 2 mM L-glutamine (Gibco), 50 mM HEPES (Gibco), 35 U/ml penicillin (Gibco) and 35 μ g/ml streptomycin (Gibco) (Tavares et al., 2005). The parasites (1×10^6 /ml) in the logarithmic phase were incubated with a serial range of *cis*-DDP concentrations (0.25–64 μ M) during 72 h at 27 °C.

L. infantum axenic amastigote forms were grown at 37 °C with 5% CO₂ in a cell free medium called MAA/20 (medium for axenic amastigotes growth). MAA/20 consisted of modified medium 199 (Gibco) with Hank's Balanced Salt Solution supplemented with 0.5% trypto-casein (Oxoid), 15 mM D-glucose (Sigma), 5 mM glutamine (Gibco), 4 mM NaHCO₃, 0.023 mM bovine hemin (Fluka), and 25 mM HEPES (Gibco) to a final pH of 6.5 and supplemented with 20% heat inactivated fetal bovine serum (Gibco) (Tavares et al., 2005). The parasites (1×10^6 /ml) were incubated with a serial range of *cis*-DDP concentrations (0.25–64 μ M) during 72 h at 37 °C with 5% CO₂. The growth and the number of viable parasites, promastigotes and axenic amastigotes were determined by adding propidium iodide (PI) at 4 μ g/ml. Counting the number of parasites was done by flow cytometry analysis during a period of 53 s after a 1:100 cell suspension dilution in phosphate saline buffer (PBS, pH 7.4). The percentage of growth inhibition was

calculated as $(1 - (\text{growth rate of the experimental culture} / \text{growth rate of the control culture})) \times 100$. The IC_{50} , that is the concentration of the drug required to inhibit the growth at 50%, was determined by linear regression analysis.

L. infantum intracellular amastigotes were cultured in Balb/c peritoneal macrophages at 37 °C, 5% CO_2 in RPMI medium (Gibco) supplemented with 10% of heat inactivated fetal bovine serum (FBS-Gibco), 2 mM L-glutamine (Gibco), 35 U/ml penicillin (Gibco) and 35 µg/ml streptomycin (Gibco). The peritoneal macrophages were collected with 5 ml of ice cold PBS and seeded at 4×10^5 per well in a Lab Tek chamber slide and left at least 2 h at 37 °C with 5% CO_2 to become adherent. The cells were infected with stationary phase promastigotes (five parasites to one macrophage) during 4 h at 37 °C with 5% CO_2 . Non-internalized parasites were removed and the infected cells were cultured overnight at 37 °C, 5% CO_2 and then incubated with a serial range of *cis*-DDP concentrations (0.625–10 µM) during 72 h. At the end of the incubation time, the cells were washed with warmed PBS, fixed with methanol and Wright stained (Hirji et al., 1998). Intracellular amastigote burdens were microscopically assessed and the results are expressed as percent reduction of parasite burden compared to that of untreated control wells. Control non-infected peritoneal macrophages were incubated with *cis*-DDP until 10 µM for 72 h showed 100% of viability by the MTT assay.

2.3. Cell cycle analysis

The cell cycle analysis of both promastigote and axenic amastigote forms was performed according to Vaisman et al. (1997) with minor modifications. Briefly, after 24 h of *cis*-DDP treatment, cells were collected and washed in ice-cold PBS and incubated overnight in 70% ethanol at 4 °C. The cells were then treated with 100 mg/ml RNase A (final concentration) and 10 mg/ml propidium iodide (final concentration) overnight at 4 °C. The cellular DNA content was analyzed using a FACSCalibur (Becton Dickinson Biosciences). The percentage of cells in G1, S and G2 phases were obtained by analyzing 3×10^4 nuclei for each experimental sample, using Cell Quest Pro and ModFitt for acquisition and analysis, respectively.

2.4. In situ TUNEL assay

DNA fragmentation was analyzed *in situ* using a fluorescent detection system (*In situ* Cell Death Detection Kit, Fluorescein-Roche). Cell suspensions at 2×10^7 /ml,

of untreated and drug treated promastigotes and axenic amastigotes with 10 and 30 µM of *cis*-DDP during 24 h, were fixed for 1 h with 2% paraformaldehyde and washed with PBS, pH 7.4. The TUNEL assay was carried out following the manufacturer's instructions and the cells were analyzed using FLH-1 detector on the Cell Quest Pro software from a FACSCalibur (BD Biosciences).

2.5. Flow cytometry analysis of DNA content

The DNA content of promastigotes and axenic amastigotes (1×10^6 /ml), treated or untreated with several concentrations of *cis*-DDP for 24 h, was determined using 4 µg/ml of propidium iodide after cell permeabilization with 2% saponin (Sigma) in PBS. Cells were analyzed using FLH-3 detector on the Cell Quest Pro, software from a FACSCalibur, (BD Biosciences).

2.6. Measurement of mitochondrial membrane potential in live *Leishmania* promastigotes and axenic amastigotes

Tetramethylrhodamine ethylester perchlorate (TMRE, Molecular Probes) is a cationic lipophilic dye that accumulates in the negatively charged mitochondrial matrix according to the Nernst equation potential (Ehrenberg et al., 1988). A stock of TMRE was prepared at in DMSO (4 mg/ml) and stored at –20 °C. For determination of the mitochondrial membrane potential, *cis*-DDP treated or untreated *L. infantum* promastigotes and axenic amastigotes were washed once in PBS and resuspended in PBS (2×10^6 cells/ml) containing 100 nM of TMRE. Cells were incubated for 30 min at 27 °C (promastigotes) or 37 °C (amastigotes) and analyzed by flow cytometry on the FL2-H channel. PI at 4 µg/ml was used to exclude dead cells and the fluorescence was recorded on FL3-H channel.

2.7. Reactive oxygen species measurement

The reactive oxygen species (ROS) production was measured using 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA) (Molecular Probes, Invitrogen). This compound is an uncharged cell permeable molecule. Inside the cells, the probe is cleaved by non-specific esterases, forming carboxydichlorofluorescein, which is oxidized in the presence of ROS (H₂O₂, OH•, HOO• and ONOO[–]). The promastigotes treated with *cis*-DDP for 24 h were washed with PBS and loaded with 10 µM of the CM-H₂DCFDA in PBS for 30 min at 27 °C. The positive control was achieved by a pre-treatment of the

parasites with 1 mM of H_2O_2 at 27 °C during 3 h. Fluorescence was monitored using a FACSCalibur, at 485 nm and 538 nm for excitation and emission, respectively.

2.8. Total glutathione and GSSG measurement

The GSH and GSSG contents of promastigotes and axenic amastigotes after *cis*-DDP treatment were determined by the DTNB–GSSG reductase recycling assay as previously described (Anderson, 1985) with some modifications. Briefly, after incubation with 0 μM , 10 μM or 30 μM of *cis*-DDP for 24 h, the parasites were collected and washed three times with ice cold PBS. The parasite pellet corresponding to each 5×10^7 parasites were precipitated with 100 μl of 5% perchloric acid and centrifuged for 5 min at 13 000 rpm in a refrigerated centrifuge (4 °C). The supernatant was collected to determine the GSH and GSSG content and the pellet resuspended in 0.3 M NaOH to determine the protein concentration. Each 100 μl acidic supernatant was neutralized with 100 μl 0.7 M KHCO_3 and the sample centrifuged for 1 min at 13 000 rpm. Fresh reagent, containing 0.24 mM NADPH and 0.7 mM DTNB (Sigma) in 72 mM phosphate buffer, was daily prepared. For measurement of total glutathione, 100 μl /well of samples, standards or blank were added in triplicate to 96-well microtiter plates, followed by 65 μl /well of the freshly prepared reagent. Plates were then incubated in a plate reader (PowerWave XS; Bio-Tek) at 30 °C for 15 min prior to the addition of 40 μl glutathione reductase per well (7 U/ml in phosphate buffer). The stoichiometric formation of 5-thio-2-nitrobenzoic acid (TNB) was followed for 3 min at 415 nm and compared with a standard curve. For the determination of GSSG, 100 μl aliquots of acidic supernatant were treated with 5 μl of 2-vinylpyridine and mixed continuously for 1 h for derivatization of GSH. GSSG was then measured as described above for total glutathione.

2.9. Statistical analysis

The data were analyzed using Student's *t*-test.

3. Results

3.1. In vitro effect of *cis*-DDP on promastigotes, axenic and intracellular amastigotes

Treatment of *L. infantum* promastigotes and axenic amastigotes (0.25–64 μM of *cis*-DDP for 72 h) showed a dose dependent inhibition of parasite growth (Fig. 1). It can be seen that concentrations $\geq 16 \mu\text{M}$ *cis*-DDP

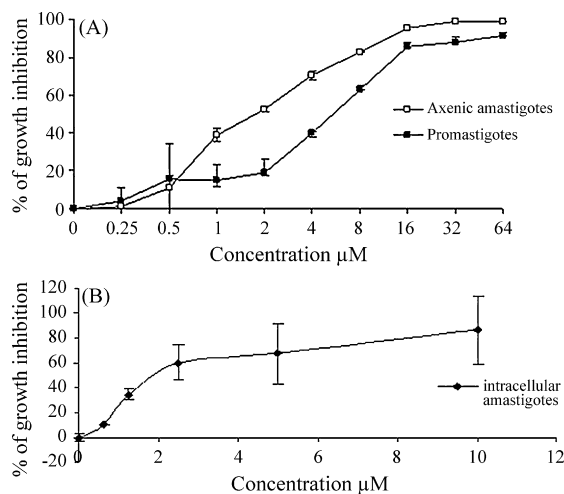


Fig. 1. *In vitro* effect of *cis*-DDP on parasite growth. Representative inhibition growth curves of promastigotes and axenic amastigotes (A). Promastigote (closed square) and amastigote (open square) forms were incubated with a range of *cis*-DDP concentrations 0.25–64 μM during 3 days at 27 °C and 37 °C, 5% CO_2 , respectively. Percentages of promastigote and amastigote growth inhibition were determined by flow cytometry counting of the viable parasites. Each point represents the mean of three replicates $\pm$ the standard deviation. Representative inhibition growth curve of intracellular amastigotes (B) cultured in mouse peritoneal macrophages incubated with a range of *cis*-DDP concentrations 0.625–10 μM during 3 days at 37 °C in 5% CO_2 . The growth inhibition percentages were determined based on the parasite burden compared to that of untreated control cells that were microscopically assessed. Each point represents the mean of two replicates $\pm$ the range. The data shown is representative of at least three independent experiments.

inhibited $\cong 90\%$ of promastigote and axenic amastigotes growth. $\text{IC}_{50} \pm \text{S.D.}$ values after 72 h drug incubation were determined to be $7.73 \pm 1.03 \mu\text{M}$, $1.88 \pm 0.10 \mu\text{M}$ and $1.85 \pm 0.22 \mu\text{M}$, for promastigotes, axenic and intracellular amastigotes, respectively. Interestingly, the amastigote, the vertebrate stage of the parasite, appear to be highly sensitive to the *cis*-DDP induced growth arrest. Our data differ from those reported recently by Nguewa et al. (2005). The latter had shown that *cis*-DDP had a cytotoxic activity against *L. infantum* promastigotes with an IC_{50} value of $150 \pm 14 \mu\text{M}$, although the effect on amastigotes was not evaluated. Although, the reason of this apparent discrepancy is unknown, one explanation could be the appearance of drug resistance in the strain used by Nguewa et al. (2005). It is noteworthy to mention that human pancreatic cell lines were shown to be less sensitive than 50 μM of the same *cis*-DDP lot as the one used in our study (Ouassii, 2004). Moreover, other investigators have shown that the human melanoma cell line 224 and the same enucleated cell line as well as HCT 116 human colon cancer cells were sensitive to *cis*-DDP

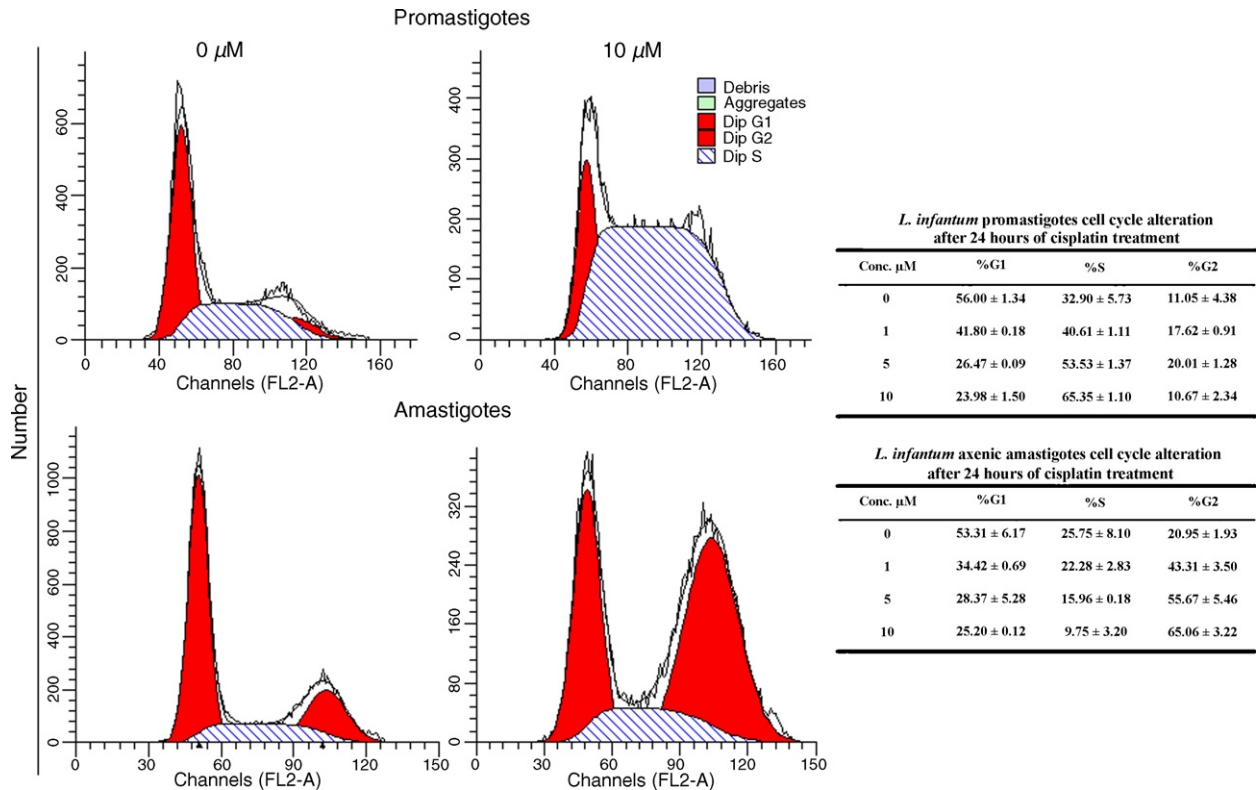


Fig. 2. Effect of *cis*-DDP on *L. infantum* promastigotes and axenic amastigotes cell cycle. The promastigotes and axenic amastigotes were treated with 1 μM , 5 μM and 10 μM of *cis*-DDP during 24 h in order to analyze the effect on the cell cycle. The cells were analyzed on the FACScan flow cytometry and the cell cycle was obtained using the Modfit software. The results are representative of two independent experiments and given as the mean $\pm$ standard deviation of an experiment performed in duplicate.

inducing apoptosis with less than 20 μM (Mandic et al., 2002, 2003).

Based on the above toxicity results, detailed time-course and dose response analyses of cell cycle changes in *L. infantum* promastigotes and axenic amastigotes exposed to *cis*-DDP were carried out. Significant increase in the population of S-phase promastigotes was observed whereas treated amastigotes accumulated in the G2-phase by 12 h (data not shown) and 24 h (10 μM *cis*-DDP) (Fig. 2). The observed increased population of promastigotes and amastigotes in the S and G2 phase, respectively, could be seen in the concentration range (1–10 μM) that includes the IC_{50} for both forms.

3.2. Parasite stage-dependent alteration of DNA by *cis*-DDP

The cell cycle arrest induced by *cis*-DDP treatment prompted us to examine the nuclear changes occurred in drug treated parasites. These were undertaken by the TUNEL assay which detects the free ends of DNA after breakage.

Promastigotes and axenic amastigotes incubated with 10 μM or 30 μM of *cis*-DDP compound for 24 h at 27 $^{\circ}\text{C}$ and 37 $^{\circ}\text{C}$, respectively, showed a *cis*-DDP dose-dependent increase in the percentage of TUNEL positive cells only in the case of promastigotes when compared to the non-treated cells. Although the positive signal was lower than that observed with parasites incubated with DNase, it remains significantly distinguishable (Fig. 3A). All the parasite samples treated with *cis*-DDP or DNase showed bright fluorescent nuclei indicating fragmentation of DNA (Fig. 3B). However, examination of a number of slides showed a slightly different pattern of labeling. Indeed, the drug induced labeling on the mitochondrial DNA (kinetoplast) was visible after 24 h of incubation. Moreover, only moderate fluorescent signal could be seen in the case of parasite nuclei when compared to kinetoplast (Fig. 3B). As a positive control, parasite samples treated with DNase showed that almost all the nuclei and kinetoplasts were fluorescent (Fig. 3B).

Since previous studies have shown that apoptosis-like death in *Trypanosome* and *Leishmania* parasites is asso-

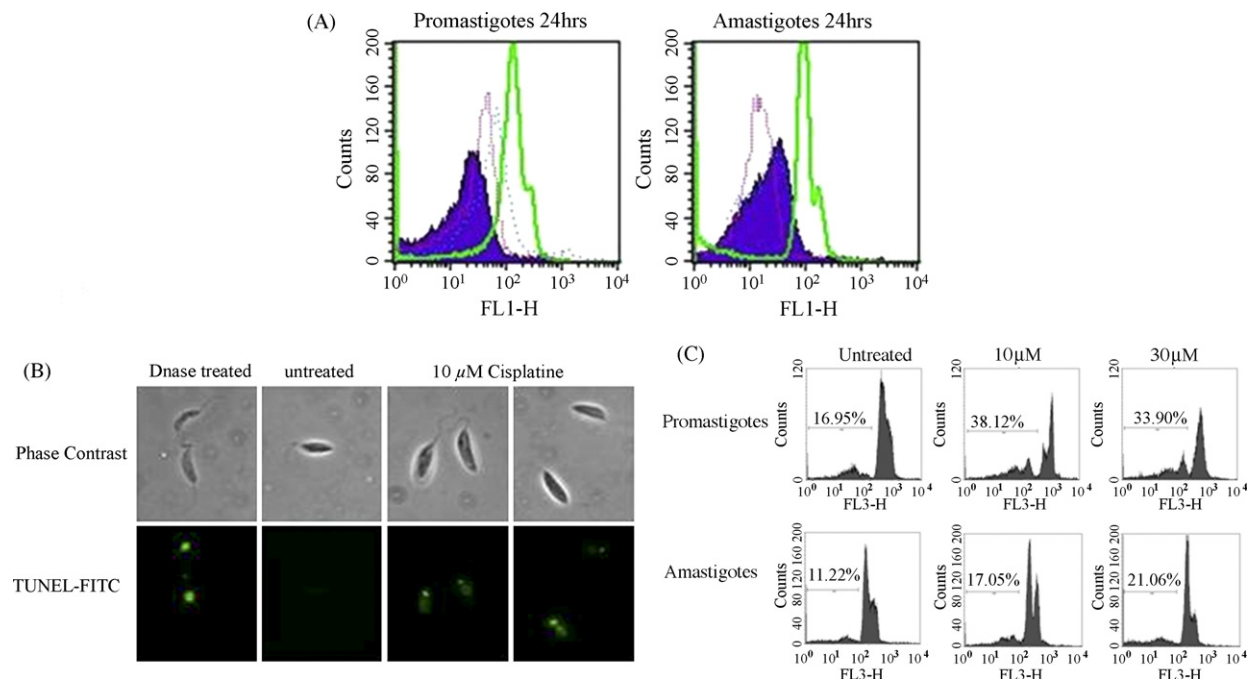


Fig. 3. Analysis of *L. infantum* promastigotes and axenic amastigotes DNA fragmentation. TUNEL assay (TUNEL, FITC) was performed on logarithmic phase promastigotes and axenic amastigotes. Untreated parasites (full peak, blue, violet), DNase treated (hatched line, green), 10 μ M (continuous line, pink) and 30 μ M (dot line, blue) of *cis*-DDP treatment during 24 h in promastigotes and amastigotes. The extent of the label was analyzed by flow cytometry (A) and fluorescence microscopy (B). Twenty four hour treated promastigotes were analyzed under a fluorescent microscope (Axioskop-Carl Zeiss, Germany) at 1000 $\times$ magnification and images captured with a digital camera (Spot 2, Diagnostic Instruments, USA) and the software Spot 3.1 (Diagnostic Instruments, USA). The DNA content of the treated promastigotes and axenic amastigotes with 10 μ M and 30 μ M of *cis*-DDP were analyzed by flow cytometry (C). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

ciated with DNA fragmentation in nucleosome-sized DNA fragments, we tested for the presence of this feature in genomic DNA samples from drug treated parasites and were unable to demonstrate any typical oligonucleosomal fragmentation (data not shown) (Ameisen et al., 1995; Lee et al., 2002).

Flow cytometry analysis after cell permeabilization and labeling with PI was carried out in order to quantify the percentage of pseudohypodiploid cells. The amount of bound dye is correlated with the DNA content in a given cell, and DNA fragmentation in apoptotic cells translates into a fluorescence intensity lower than that of G1 cells (sub-G1 peak) (Nicoletti et al., 1991). After 24 h of promastigotes and axenic amastigotes incubation with 10 μ M or 30 μ M of *cis*-DDP, the percentages of cells found in the sub-G1 peak region were as follow: 38.1% and 33.9% for promastigotes, and 17.1% and 21.1% in the case of amastigote forms compared with 16.9% and 11.2% in control cells, respectively (Fig. 3C), suggesting therefore, that *cis*-DDP induced DNA degradation in promastigotes.

3.3. Quantification of phosphatidylserine externalization in *L. infantum* promastigote and amastigote forms treated with *cis*-DDP

In mammalian cell apoptosis, the translocation of phosphatidylserine occurs from the inner side to the outer layer of the plasma membrane. Annexin V, a Ca^{2+} dependent phospholipids-binding protein with affinity for phosphatidylserine, is routinely used in a fluorescein-conjugated form to label externalization of phosphatidylserine. Since annexin V-FITC can also label necrotic cells following the loss of membrane integrity, simultaneous addition of PI, which does not permeate cells with an intact plasma membrane, allows discrimination between apoptotic cells (annexin V positive and PI negative), necrotic cells (annexin V positive and PI positive) and live cells (annexin V negative and PI negative). Amastigote and promastigote forms from *L. infantum* were incubated with increasing concentrations of *cis*-DDP (1–30 μ M) for 24 h at 37 $^{\circ}\text{C}$ and 27 $^{\circ}\text{C}$, respectively, and processed for phosphatidylserine externalization measurement. The percentages of annexin

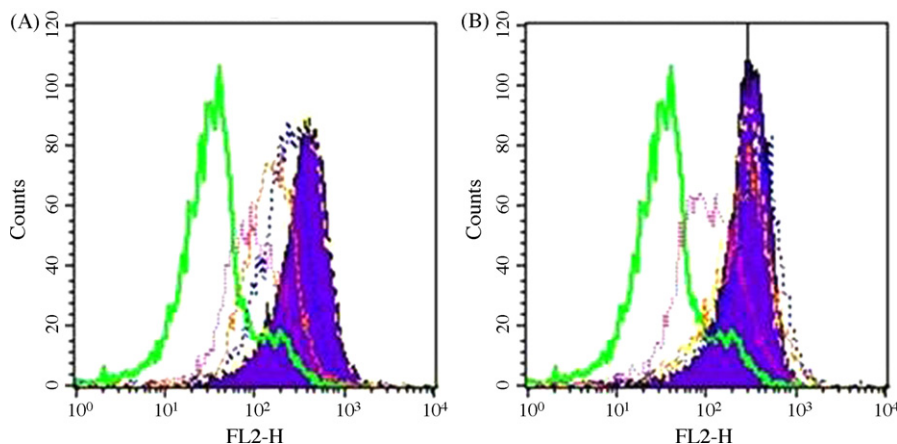


Fig. 4. Modification of mitochondrial membrane potential by *cis*-DDP. Live *Leishmania* promastigotes (A) or axenic amastigotes (B) were treated with 30 μ M (pink line), 10 μ M (orange line), 5 μ M (blue line) and 1 μ M (yellow line) of *cis*-DDP for 24 h. Untreated cells (full peak) showed positive control labeling and the uncoupler CCCP (continuous line) showed negative control labeling. The histogram shows fluorescence intensity of TMRE after 30 min of fluorescent probe incubation. The results are representative of one experiment out of three performed in duplicate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

positive-parasites slightly increased from 0.6% to 2.6% in the case of promastigotes when compared to non-treated cells (0.6%) and from 3.3% to 5.1% in the case of amastigotes when compared to non-treated ones (2.4%) or to parasites UV-irradiated showing 90.0% positive cells (data not shown).

3.4. *L. infantum* cell death induced by *cis*-DDP is associated with the modification of mitochondria membrane potential

Previous studies suggested that nuclear features of apoptosis in metazoan cells, like condensation of nuclei

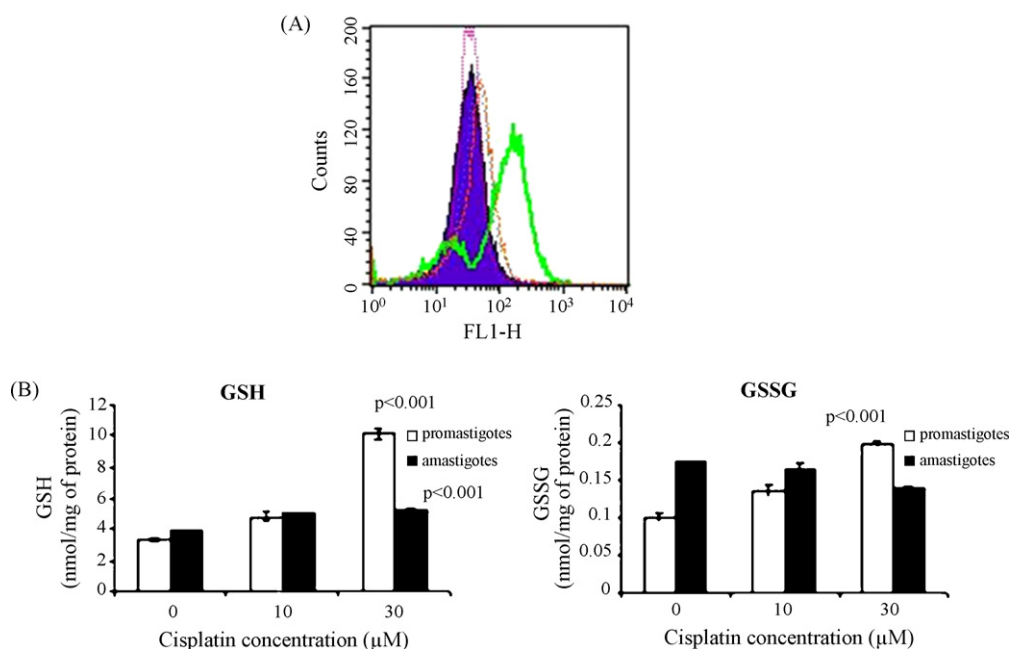


Fig. 5. The role of *cis*-DDP on the oxidative stress. The reactive oxygen species (ROS) production was measured using the CM-H₂DCFDA on *L. infantum* promastigote after 24 h of treatment with 5 μ M (hatched line, pink), 10 μ M (dot line, blue) and 30 μ M (hatched and dot line, orange) of *cis*-DDP (A). The positive labeling (thick continuous line, green) was achieved by incubating the promastigotes with 1 mM of H₂O₂ during 3 h and the negative (full peak, blue) was given by the untreated cells. The GSH and GSSG levels were determined after 24 h of promastigotes and axenic amastigotes treatment with 10 μ M and 30 μ M of *cis*-DDP (B). The results are representative of three independent experiments performed in triplicate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

and fragmentation of DNA, are preceded by alterations in mitochondrial structure and transmembrane potential (Zamzami et al., 2001). Thus, we explored whether *cis*-DDP treatment caused an early response in the parasite mitochondrial membrane potential.

To measure the mitochondrial membrane potential TMRE fluorescent probe combined to PI (in order to exclude dead cells) and FACS analysis were used. Thus, promastigote and axenic amastigote forms were treated during 24 h with the drug at concentrations ranging from 1 μ M to 30 μ M and then incubated with TMRE for 30 min. The histogram analysis showed a dose-dependent decrease in promastigotes (Fig. 4A) and amastigotes (Fig. 4B) mitochondrial membrane potential.

3.5. Role of *cis*-DDP in the oxidative stress

Reactive oxygen species (ROS) emanate from mitochondrial and non-mitochondrial enzymes (Kamata and Hirata, 1999). To investigate whether the ROS are involved in *cis*-DDP induced parasite death we determined the levels of ROS produced after 24 h in *cis*-DDP treated promastigotes at 5 μ M, 10 μ M and 30 μ M concentrations and CM-H₂DCFDA labeling (Fig. 5A). Production of ROS in promastigotes is lower than the one observed in H₂O₂ treated cells. Detectable amount of ROS is only observed at *cis*-DDP concentrations higher than 10 μ M. In contrast, the ROS were undetectable in the case of drug-treated amastigotes. Surprisingly, this was also the case when using H₂O₂ treated amastigotes as a possible positive control.

Since the levels of ROS detected in drug treated parasites were lower than expected we decided to measure the GSH/GSSG levels. As shown in Fig. 5B, 24 h *cis*-DDP 30 μ M treated promastigotes showed highly significant increase of intracellular GSH and GSSH levels when compared to untreated parasites and even with amastigotes.

4. Discussion and conclusion

The antiparasitic properties of the classical anticancer agent, *cis*-DDP, was analyzed and characterized. We report that this compound is able to inhibit the growth of all the *L. infantum* stages in a dose dependent manner and was more active against the mammalian stage. The DNA is thought to be the critical target for this type of compounds although Mandic et al. (2003) has clearly demonstrated that *cis*-DDP is able to induce nucleus independent apoptotic signaling. The formation of Pt adducts has been shown to block DNA replication and

consequently induce growth arrest. In the present study we demonstrated that *cis*-DDP induced a stage dependent cell cycle arrest being the promastigotes and axenic amastigotes blocked at the S and G2 phase, respectively. However, the DNA damage was only detected in the case of promastigotes. The G2 block has been proposed to be a major check point for damaged cells (Vaisman et al., 1997) and cells emerging from the G2 arrest could progress through the cell cycle, that depend on the extent which the DNA damage can be repaired during G2 arrest. We can speculate that the absence of DNA damage in the case of the amastigotes could be due to the repair that occurs during G2 arrest (Ormerod et al., 1994; Sorenson and Eastman, 1988). In addition to the *cis*-DDP effects on DNA, other mechanisms like inhibition of protein synthesis and mitochondrial damage have been proposed (Huang et al., 1995). One explanation is related to the fact that in aqueous solutions the chloride ligand of *cis*-DDP is replaced by water molecules generating a positively charged electrophile that can react with glutathione and the resultant complex transported inside the mitochondria via one of the known glutathione transporters (Dzamitika et al., 2006).

Our study also suggests that *Leishmania* mitochondria might be a non-nuclear target of *cis*-DDP. Indeed, *cis*-DDP treatment leads to a partial disruption of the mitochondrial membrane potential and functional alterations.

Moreover, *cis*-DDP has been shown to induce oxidative stress in a variety of cell lines (Das et al., 2006; Kawai et al., 2006; Sato et al., 2006) and the treatment with ROS scavenger, *N*-acetylcysteine, resulted in decreased nuclear fragmentation and mitochondrial depolarization (Mandic et al., 2003). In the case of *Leishmania* parasites, the role of ROS has a limited function with a low level being detected in promastigote forms after *cis*-DDP treatment. The increase in GSH levels has been described as one of the mechanisms involved in cell resistance to *cis*-DDP (Jansen et al., 2002). Treatment of *Leishmania* promastigotes with *cis*-DDP resulted in significant increase of GSH levels and this might explain the higher IC₅₀ observed for this parasite stage.

Overall, these results suggest that the *cis*-DDP, an anticancer drug, exert an anti-parasitic activity with different mode of actions against *Leishmania* developmental stages.

Screening compounds with known toxic effects against *Leishmania* is a useful approach in enhancing our knowledge of the biological events that regulate the processes of growth arrest and death in this parasite.

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2.2 Targeted disruption of cytosolic SIR2 deacetylase discloses its essential role in *Leishmania* survival and proliferation

Proteins of the SIR2 family are characterized by a conserved catalytic domain that exerts unique NAD-dependent deacetylase activity on histone and various other cellular substrates. Functional analyses of such proteins have been carried out in a number of prokaryotes and eukaryotes organisms but until now, none have described an essential function for any SIR2 genes. Here using genetic approach, we report that a cytosolic SIR2 homolog in *Leishmania* is determinant to parasite survival. *L. infantum* promastigote tolerates deletion of one wild-type LiSIR2 allele (LiSIR2^{+/-}) but achievement of null chromosomal mutants (LiSIR2^{-/-}) requires episomal rescue. Accordingly, plasmid cure shows that these parasites maintain episome even in absence of drug pressure. Though single LiSIR2 gene disruption (LiSIR2^{+/-}) does not affect the growth of parasite in the promastigote form, axenic amastigotes display a marked reduction in their capacity to multiply *in vitro* inside macrophages and *in vivo* in Balb/c mice. Taken together these data support a stage specific requirement and/or activity of the *Leishmania* cytosolic SIR2 protein and reveal an unrelated essential function for the life cycle of this unicellular pathogenic organism. The lack of an effective vaccine against leishmaniasis, and the need for alternative drug treatments, makes LiSIR2 protein a new attractive therapeutic target.

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Targeted disruption of cytosolic SIR2 deacetylase discloses its essential role in *Leishmania* survival and proliferation

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Abstract

Proteins of the SIR2 family are characterized by a conserved catalytic domain that exerts unique NAD-dependent deacetylase activity on histone and various other cellular substrates. Functional analyses of such proteins have been carried out in a number of prokaryotes and eukaryotes organisms but until now, none have described an essential function for any *SIR2* genes. Here using genetic approach, we report that a cytosolic SIR2 homolog in *Leishmania* is determinant to parasite survival. *L. infantum* promastigote tolerates deletion of one wild-type *LiSIR2* allele (*LiSIR2*^{+/−}) but achievement of null chromosomal mutants (*LiSIR2*^{−/−}) requires episomal rescue. Accordingly, plasmid cure shows that these parasites maintain episome even in absence of drug pressure. Though single *LiSIR2* gene disruption (*LiSIR2*^{+/−}) does not affect the growth of parasite in the promastigote form, axenic amastigotes display a marked reduction in their capacity to multiply *in vitro* inside macrophages and *in vivo* in Balb/c mice. Taken together these data support a stage specific requirement and/or activity of the *Leishmania* cytosolic SIR2 protein and reveal an unrelated essential function for the life cycle of this unicellular pathogenic organism. The lack of an effective vaccine against leishmaniasis, and the need for alternative drug treatments, makes *LiSIR2* protein a new attractive therapeutic target.

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Keywords: *Leishmania infantum*; Gene disruption; *LiSIR2*; Cell cycle; Virulence

1. Introduction

SIR2 (Silent Information Regulator 2) genes have been cloned from a wide variety of organisms ranging from bacteria to

human. All share a common conserved core domain of about 250 residues responsible for a deacetylase activity capable of removing the acetyl moiety from the ε-amino group of lysine residues in protein substrate. These enzymes couple deacetylation to the hydrolysis of NAD⁺, transferring the acetyl group from substrate to ADP-ribose, thereby generating a novel compound, O-acetyl-ADP-ribose (Tanny and Moazed, 2002; Tanner et al., 2000). Five *SIR2* related proteins are present in yeast (*ySIR2*, *HST1*–4) and seven in human (*SIRT1*–7). Diverse subcellular localizations among *SIR2* homologs have been described. Some are present in the nucleus, while others seem to be mainly cytoplasmic, like the yeast Hst2p (Perrod et al., 2001), the *Leishmania* *SIR2* proteins (Zemzoumi et al., 1998) or the human *SIRT2* (North et al., 2003; Afshar and Murnane, 1999). A

Abbreviations: *LiSIR2*, *Leishmania infantum* Silent Information Regulatory gene homologue; pXG-BSDLiSIR2 plasmid, plasmid which confers resistance to Blasticidin and carrying the *LiSIR2* gene; PFGE, Pulse field gel electrophoresis; NAD, nicotinamide-adenine dinucleotide; HDAC, histone deacetylase; WT, wild type parasite clone; neo and hyg, the neomycin phosphotransferase and the hygromycin phosphotransferase cassettes, respectively; ORF, open reading frame.

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mitochondrial localization has also been described for the human SIRT3 (Onyango et al., 2002). A large number of studies focusing on SIR2p with exclusive nuclear localization pattern have demonstrated a conserved role in the regulation of aging process in yeast, *C. elegans* (Tissenbaum and Guarente, 2001; Kaeberlein et al., 1999) or more recently in metazoans (Wood et al., 2004). Even if the implication in ageing process seems to be evolutionary conserved, it involves different pathways suggesting that biological properties of SIR2 proteins have diverged during evolution. Little is known about putative function(s) of cytoplasmic SIR2 proteins, though the implication of the human SIRT2 in control of mitosis, likely through its tubulin deacetylase activity, has been proposed (Dryden et al., 2003; North et al., 2003). Recently, SIRT2 has also been shown to interact with the homeobox transcription factor HOXA10 raising a probable involvement of SIRT2 in mammalian development (Bae et al., 2004). In fact, studies on mammalian SIRT1 and SIRT2 proteins pointed out that SIR2 proteins may have several physiological targets or partners and thus could be part of different biological processes. However, as the number of SIR2 gene-related family members is increasing, functional studies are required to analyze the biological properties of the different SIR2 entities. Nevertheless none of SIR2 genes have yet been reported to be essential (Astrom et al., 2003; Smith et al., 2000).

Leishmania are protozoan parasites that are causative agents of several medically important tropical diseases concerning more than 350 millions of peoples over 88 countries around the world. They are transmitted by a blood feeding dipteran vector of the sub-family *Phlebotominae*. These parasites exist as flagellated promastigotes within sandfly vectors and as intracellular amastigotes residing inside macrophages of infected vertebrate hosts (Grimaldi and Tesh, 1993). Experimentally generated genetically modified parasite clones exhibiting various biological phenotypes have been used to analyze *Leishmania* virulence factors (Beverley, 2003). In more recent experiments such approach has been used to define parasite factors affecting the persistence of the pathogen in its vertebrate host and their role in disease progression (Spath et al., 2003). In our search for SIR2 homologs, we have characterized SIR2 proteins from *L. major* (*LmSIR2*) (Yahiaoui et al., 1996) and *L. infantum* (*LiSIR2*) (Vergnes et al., 2002) while two other related sequences can be found in the *Leishmania* genome database (*L. major* sirtuin (CAB55543), *L. major* cobB (LmjF34.2140)). Indirect molecular and biological approaches allowed us to show the potential role of *LmSIR2* and *LiSIR2* genes in parasite survival, due to an inherent resistance to apoptosis-like death (Vergnes et al., 2002). In this study we used a “reverse” genetic approach to probe biological function(s) of the *L. infantum* cytosolic SIR2 protein in the parasite infectious cycle.

2. Materials and methods

2.1. Parasite culture and experimental infections of THP-1 derived macrophages

Promastigotes of the *Leishmania infantum* (clone MHOM/67/ITMAP-263) and derived mutants clones were obtained by

limiting dilution and grown at 26 °C in SDM-79 medium (Brun and Schonenberger, 1979) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin. Clonal populations of axenically grown amastigotes forms were maintained at 36±1 °C with 5% CO₂ in a cell free medium called MAA/20 (Sereno and Lemesre, 1997). Infection of THP-1 derived macrophages with axenic amastigotes was carried out as outline earlier (Sereno et al., 1998).

2.2. DNA constructs

2.2.1. Targeting constructs

A *L. infantum* genomic fragment containing the *LiSIR2* gene and its flanking regions was isolated by screening a parasite genomic library (Kindly provided by Dr M. Ouellette) using the *LmSIR2* (*Leishmania major* SIR2) gene sequence as a probe. The α(³²P)dCTP labeled probe was used to screen the genomic library by plaque filter hybridization, according to the manufacturer's instructions (Stratagene). Positive clones were subjected to restriction map and Southern blot analysis with the same probe. A reactive *Hind*III fragment of 5.9 Kb was isolated and subcloned into pUC18 plasmid vector (Amersham) and sequenced (pUCSIR2 plasmid). ORF determination revealed a sequence of 1119 bp encoding a protein of 373 aa sharing 93% overall identity with the *L. major* *LmSIR2* encoding sequence. The pUCSIR2 plasmid was then used to target both *LiSIR2* alleles. The neomycin phosphotransferase (neo) and the hygromycin phosphotransferase (Hyg) expression cassettes derived from pSPYneo and pSPYhyg respectively (Papadopoulos et al., 1994) were both isolated by *Bam*HI–*Bgl*III digestion and treated with klenow fragment to produce blunt ends. pUCSIR2 plasmid was opened by the unique *Cla*I site within the *LiSIR2* ORF and treated successively with Mug bean nuclease and shrimp phosphatase to generate dephosphorylated blunt ends. Targeting vectors pUCSIR2neo and pUCSIR2hyg were then generated by introducing either the neo or the hyg cassettes into the *Cla*I linearized pUCSIR2 vector. Linear targeting regions produced by digesting either pUCSIR2neo or pUCSIR2hyg by *Hind*III were gel purified twice and used sequentially to transfect mid-log phase *L. infantum* promastigotes.

2.2.2. pXG-BSD *LiSIR2*

Plasmid rescue experiments were carried out using the pXG-BSD plasmid (kindly provided by Dr Beverley) which confers resistance to Blasticidin S (Goyard and Beverley, 2000). Sequencing of the pUCSIR2 vector allow us to design primers used to clone *LiSIR2* gene within pCR2.1 plasmid (TA cloning, Invitrogen). *LiSIR2* coding sequence obtained by *Eco*RI digestion was treated by Mug Bean nuclease to obtain blunt ends and ligated into the *Sma*I site of linearized dephosphorylated pXG-BSD vector. Right orientation was then screened by restriction enzymes.

Oligonucleotides used in this study: *LiSIR2* Forward 5'-GGAGATGACAGCGTCTCCGAGAGC-3'; *LiSIR2* Reverse 5'-TCAGGTCTCATTCGGCGCCCTCTG-3'; *Lmsirtuin*

Forward 5'-ATGAGGCCGGCGGGGACGCTTGCA-3'; Lmsir-tuin Reverse 5'-CTAGAGTTGAATCGTCTTGCGGCG-3'.

2.3. Transfection, selection and cloning of *L. infantum* promastigotes

Mid-Log phase promastigotes were transfected at 450 V cm^{-1} and 450 μF with 1 μg of linearized agarose purified DNA as previously described (Vergnes et al., 2002). After electroporation, parasites were incubated in 5 ml of drug-free SDM-79 10% FCS medium for 24 h, after which drug(s) was added for 2 weeks: G418 (20 $\mu\text{g}/\text{ml}$), Hygromycin B (50 $\mu\text{g}/\text{ml}$), Blastidicin S (30 $\mu\text{g}/\text{ml}$). Parasites were then cloned by limiting dilution.

2.4. Plasmid cure

Single and double knock out parasites transfected with the pXG-BSD*LiSIR2* plasmid were maintained in culture by weekly subpassages in absence of selectable marker blastidicin S. At both eleven and twenty one weeks, total DNA from promastigotes was extracted and used to selectively amplify *LiSIR2* gene from the episome by using specific pXG-BSD backbone primer (5'-AAACCGCTCGCGTGTGTTGAGCCG-3') and *LiSIR2* reverse one. Furthermore, sensitivity to blastidicin S has been analyzed by measuring the growth inhibition index at day 3 of culture = $(1 - \text{parasite number with } 200 \mu\text{g}/\text{ml} \text{ blastidicin S} / \text{parasite number without blastidicin S}) \times 100$. Data are the mean of two independent experiments.

2.5. PFGE and Southern blot analysis

Late log phase parasites were harvested by centrifugation, washed twice in NaCl 0.9% buffer and mixed volume/volume with 1% Incert agarose (tebu) in TBE 1 $\times$ to a final concentration of 5.10^8 parasites/ml. Promastigotes in solidified agarose were lysed in situ by overnight treatment with 5 volumes of proteinase K 1 mg/ml in 0.5 M EDTA, 1% sodium lauryl sarcosinate at 50 °C. Plugs were washed four times for 30 min in TE buffer at room temperature and stored in TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0) at 4 °C. Chromosomes were loaded onto a 150 ml gel of 0.7% or 1.2% (w/v) standard agarose. PFGE was carried out at 70 V with a pulse ramp of 20–30 min for 96 h using a CHEF Gene Navigator apparatus (Amersham-Biosciences) in TBE 0.5 $\times$ at 9 °C. Gel was visualized in ethidium bromide and transferred by capillarity into nylon membrane Hybond-N+ (Amersham). Southern and PFGE membranes were hybridized with $\alpha\text{P}^{32}\text{dCTP}$ labelled probes as previously described (Vergnes et al., 2002).

2.6. Flow cytometry analysis using propidium iodide

Amastigote and promastigote multiplication and viability were determined using flow cytometry analysis. Briefly, sample of cells (10 μl) were daily taken from the culture and diluted into 500 μl of PBS (pH 7.2). Propidium iodide at a final

concentration of 1 $\mu\text{g}/\text{ml}$ was added and cells were analyzed on the cytometer for 1 minute and 45 s. Fluorescence gated on forward and side light scatter was collected and displayed using a logarithmic amplification (FL2–FL3). The quadrant statistic allowed us to determine the percentage of viable cells. The parasite number was determined using standard curves where parasites concentration were plotted as a function of mean cells count by cytometer after 1 min 45 s (Vergnes et al., 2002).

2.7. Test of deacetylase activity

HDAC assay was carried out essentially as described (Brehm et al., 1998) in 100 μl of NAD (nicotinamide–adenine dinucleotide)–HDAC buffer containing 50 mM Tris-HCl pH 8.8, 4 mM MgCl_2 , 0.2 mM dithiothreitol (DTT) with 75,000 cpm of a tritium labelled acetylated H4 peptide. Labelling of H4 histone peptide was carried out following manufacturer instructions (HDAC assay kit, Upstate-biotechnology). Test of deacetylase activity was performed on 1 μg of *LmSIR2* recombinant protein or 130 μg of total parasite extract from both WT and p*SIR2* overexpressing mutant parasites transfected with a pTEX-*LmSIR2* plasmid construct already described in a previous report (Vergnes et al., 2002). For inhibition experiments, 250 mM sodium butyrate was added to the reaction medium. Assays were performed in triplicate.

2.8. Experimental infections

2.8.1. In vitro macrophage infection

Phorbol myristate acetate-treated monocytes (differentiated macrophages) were incubated with stationary phase amastigotes in a parasite/cell ratio of 5:1. After 2 h of incubation, noninternalized parasites were removed and the cells incubated in a culture medium at 37 °C with 5% CO_2 up to 4 days. The cells were fixed with methanol and stained with giemsa. The number of intracellular amastigotes per cell was determined after having counted at least 800 cells per slide.

2.8.2. Balb/c infection

Seven-week-old Balb/c male mice were obtained from Harlan Iberica (Spain). The mice were infected intraperitoneally (i.p.) with 10^8 or 10^9 wild-type promastigotes of *L. infantum* (WT) and with N1 or N2 single mutant clone carrying *LiSIR2* monoallelic disruption (*LiSIR2/LiSIR2::neo*). The spleen cells from three groups of mice were submitted to the serial four-fold dilutions ranging 1 to $1/4 \times 10^{-6}$ in 96-well microtitration plates. After 15 days of incubation at 26 °C the presence or absence of motile promastigotes was recorded in each well. The final titre was the last dilution for which the well contained at least one parasite. The number of parasites per gram of spleen (parasite burden) was calculated as follows: parasite burden = (geometric mean of reciprocal titre from each triplicate cell culture/weight of homogenized spleen) $\times$ reciprocal fraction of the homogenized spleen inoculated into the first well.

3. Results

3.1. The *Leishmania* cytosolic SIR2 protein possess NAD-dependent deacetylase activity on H4 histone peptide

Given that the NAD-dependent deacetylase activity of SIR2 proteins seems to be evolutionary conserved, we firstly analyzed deacetylase activity of the *Leishmania* SIR2 protein. Although LiSIR2 protein is mainly cytosolic in parasite, we used [³H]-acetylated histone H4 peptide which is the usual substrate to test SIR2 enzymatic activity. These experiments were performed using both hexa-His-tagged fusion protein (LmSIR2rp) and total protein extract from wild type (WT) and LmSIR2 overexpressing parasites (pSIR2). As shown in Fig. 1, adding NAD to the recombinant protein led to an increasing deacetylation of histone peptide as demonstrated by the ratio of released cpm measured in presence and in the absence of NAD (ratio $\approx$ 13). Additionally, proteins extract of *L. infantum* promastigotes carrying extra copies of *LmSIR2* gene displayed a strong NAD-dependent deacetylase activity as compared to WT parasites (ratio of 11 versus 2 respectively). This deacetylase activity is not inhibited by sodium butyrate at concentration known to inhibit class I and II histone deacetylases.

3.2. Generation of *L. infantum* LiSIR2 defective parasites

To assess biological function(s) of the cytosolic SIR2 protein in *Leishmania* parasites, we tried to generate transgenic parasites lacking one or both *LiSIR2* alleles by homologous recombination. *Leishmania* are generally considered as diploid organisms with no sexual crosses that can be manipulated readily, the generation of homozygous gene knockout mutant thus requires two round of targeting with two dominant selectable markers. This was achieved by using sequentially

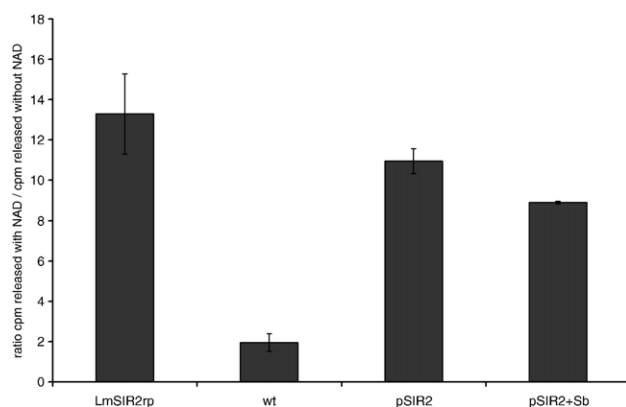


Fig. 1. Analysis of the NAD dependent HDAC activity. Histone H4 peptides were acetylated with [³H]acetic acid following the procedure supplied by the manufacturer (Upstate). The deacetylase activity was performed on 1 μ g of LmSIR2 recombinant protein (LmSIR2rp) or 130 μ g of total parasite extract from both wild type (wt) and SIR2 overexpressing mutant parasites transfected with a pTEX-LmSIR2 plasmid construct (pSIR2) already described in a previous report (Vergnes et al., 2002). Assays were performed in triplicate. Data are the mean of three independent experiments and represent the ratio of released cpm measured with and without NAD addition. 250 mM of sodium butyrate (Sb) has been added to parasite extract to inhibit class I and II HDAC activity.

two targeting constructs based on a *HindIII* genomic fragment containing the *LiSIR2* ORF, disrupted either by the *neo* or the *hyg* selectable markers (see Fig. 2A and materials and methods for details). Names and genotypes of the clones obtained during the study are indicated in Table 1. Recombination events were monitored by Southern blot analyses using either *LiSIR2*, *neo* or *hyg* specific probes. According to restriction map of the WT *LiSIR2* locus, *PstI* digestion followed by hybridization with *LiSIR2* probe will generate a unique 3.4 kb hybridization signal, whereas the presence of a second *PstI* site within the *neomycin* and *hygromycin* coding sequences will produce two signals for both *LiSIR2::neo* locus (2.6 and 2.2 kb) and *LiSIR2::hyg* locus (2.8 and 2.3 kb) (Fig. 2A). A first round of transfection with the *neo* construct allowed us to select “N clones” where one replacement event has occurred as supported by the presence of the three expected hybridization signals at 3.4, 2.6 and 2.2 kb (Fig. 2B, lane N1). Further confirmation of the *neo* construct integration was obtained by hybridization with the *neo* specific probe after *SacI* digestion (Fig. 2C, lane N1). In order to disrupt the second allele, a representative N1 clone (*LiSIR2/LiSIR2::neo*) was submitted to a second round of transfection using the *hyg* targeted construction. Parasites bearing both neomycin and hygromycin resistances were selected and named “NH” clones. Southern blot patterns of more than twenty NH clones led us to hypothesize initially that the *hyg* construct has replaced the remaining *LiSIR2* allele as evidenced by the appearance of the two expected doublets following DNA *PstI* digestion and hybridization with *LiSIR2* probe (Fig. 2B lanes NH1–3, and Fig. 2F lane NH1). Surprisingly, the *LiSIR2* probe still hybridized with a remaining WT allele at 3.4 kb. This observation suggests either that the second *LiSIR2* allele might have been duplicated or translocated elsewhere in the genome, or that the *hyg* construct has actually not been integrated in the right locus. Nevertheless, the presence of the two resistance cassettes in all NH clones analyzed was confirmed by hybridization of *SacI* digested DNA with either the *neo* or the *hyg* specific probes (Fig. 2C and D; lanes NH1–3). Additional attempt to disrupt the second allele using newly purified *hyg* targeting construct gave same results. Given that knockouts of several *SIR2* homologs were always carried out successfully on different organisms (Astrom et al., 2003; Smith et al., 2000), we then examined a potential technical bias involving our targeting constructs. Firstly, hybridization pattern obtained using the *PstI* discriminating enzyme which cut upstream of the recombination zone, exclude a direct recircularisation of the *hyg* construct, as well as a contamination with some uncut pUCSIR2hyg plasmid in the transfection mix. In order to rule out a possible defect involving the *hyg* targeting construct, we then attempted to disrupt *LiSIR2* locus by inverting the order of targeting constructs, using the *hyg* one first. As shown in Fig. 2F, single knockout “H1 clone” carrying the *hyg* construct was readily obtained. The second round of transfection using the *neo* construct allowed the production of a double hygromycin and neomycin resistant “HN clones”. However, all recombinant clones exhibit a similar ambiguous hybridization pattern than NH clones with a systematic remaining WT *LiSIR2* allele (Fig. 2F, lane HN1). Therefore, it is unlikely that technical bias

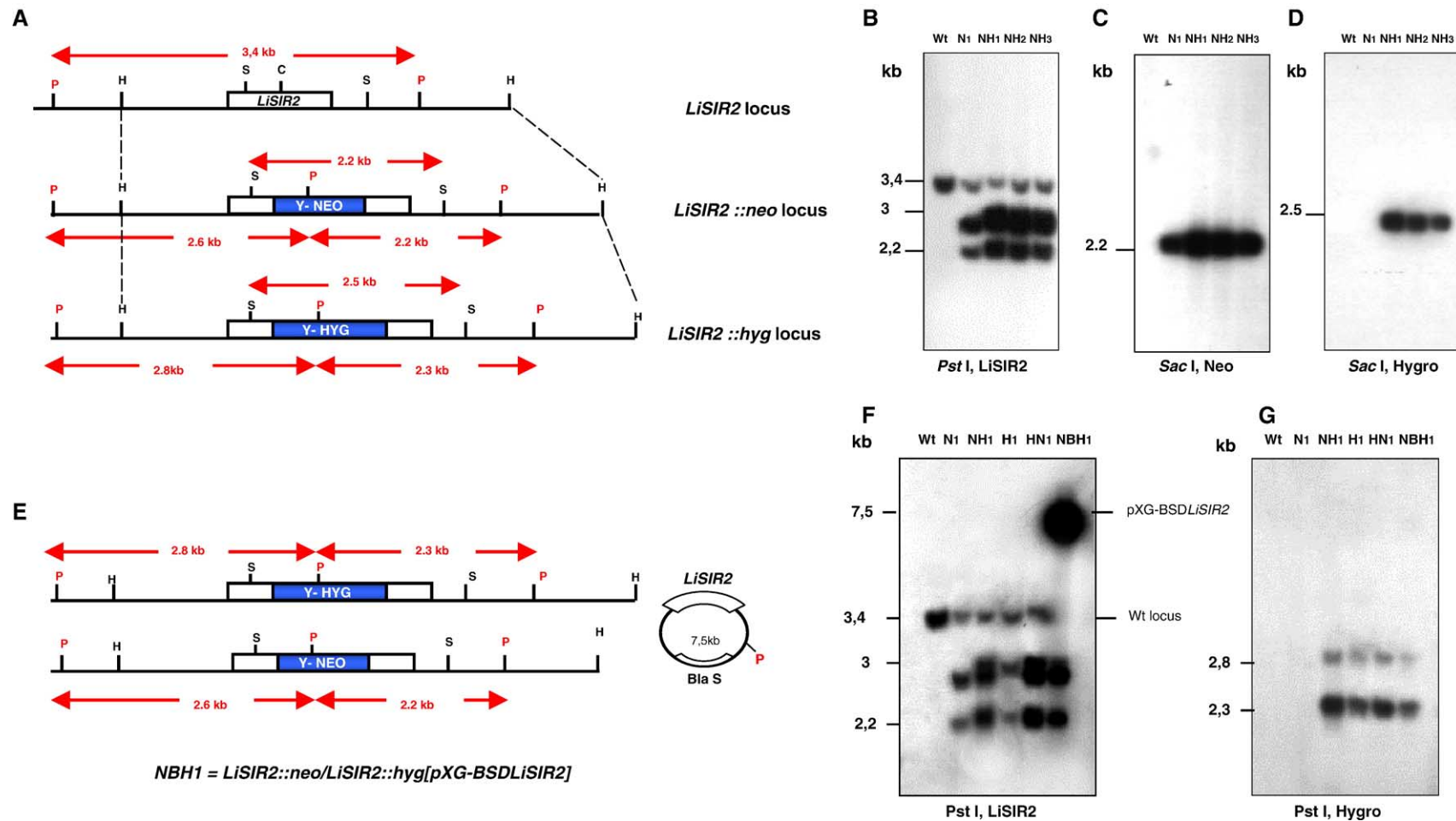


Fig. 2. (A) Schematic drawing of WT and the *neo* or *hyg* targeted *LiSIR2* locus. The *Hind*III targeted constructs and their localisation within *LiSIR2* locus are indicated by dotted line. Additional *Pst*I site within selectable markers were used to analyse recombination events. Restriction enzymes sites: P, *Pst* I; H, *Hind* III; S, *Sac* I; C, *Cla* I. (B) Southern blot analysis on selected representative clones : WT; N1 clone (*LiSIR2/LiSIR2::neo*); NH1, NH2 and NH3 (three representative *LiSIR2/LiSIR2::neo/hyg* clones). Equal amount of genomic DNA (15 µg) was digested by *Pst*I restriction enzyme and hybridized with *LiSIR2* probe. Subsequent Southern blot analyses on *Sac*I digested DNA, hybridized with *neo* (C) and *hyg* (D) probes. (E) Schematic representation of *LiSIR2* null chromosomal mutant (NBH1 clone) supplemented with pXG-BSDLiSIR2 plasmid. Southern blot analysis of *Pst*I digested DNA from the different clones obtained in this study (see Table 1 for corresponding genotypes), and hybridized with *LiSIR2* (F) and hygromycin (G) probes.

Table 1
Names and genotypes of *L. Infantum* clones used in this study

Clone names	Genotype	Drug selection
N1	<i>LiSIR2/LiSIR2::Neo</i>	Neomycin
H1	<i>LiSIR2/LiSIR2::Hyg</i>	Hygromycin
NH1, NH2, NH3	<i>LiSIR2/LiSIR2::Neo/Hyg</i>	Neomycin, hygromycin
HN1	<i>LiSIR2/LiSIR2::Hyg/Neo</i>	Hygromycin/neomycin
NB1	<i>LiSIR2/LiSIR2::Neo[pXG-BSDLiSIR2]</i>	Neomycin/blasticidin
NBH1	<i>LiSIR2::Neo/LiSIR2::Hyg[pXGBSDLiSIR2]</i>	Neomycin, blasticidin, hygromycin
pSIR2	<i>pTEX-LmSIR2</i>	Neomycin

involving either our targeted constructs or contamination of transfection mix would explain our failure to obtain true null *LiSIR2*^{−/−} mutants. These results suggest rather that a genomic rearrangement has occurred during our try to disrupt the second allele. Genomic rearrangement is indeed a well established strategy used by *Leishmania* to prevent disruption of essential genes. Those can include change in chromosomal number, gene translocation event, non-specific integration or episomal maintenance of the second targeted construct (Tovar et al., 1998; Dumas et al., 1997; Mottram et al., 1996). In our case, change of the parasite ploidy can be rule out since no differences in the DNA content was detected by FACS-scan analysis between WT and recombinant parasites (not shown). PFGE chromosome segregation, followed by hybridization was then carried out to detect a potential translocation event. Both *LmSIR2* and *LiSIR2* are known to be unique genes located on the chromosome 26 in *L. major* and *L. infantum* respectively, with an equivalent size of about 1.1 Mb (Wincker et al., 1996). As shown in Fig. 3A, when using *LiSIR2* probe, a unique signal is detected at the expected size (1.1 Mb) in both WT and *LiSIR2* mutant. Surprisingly, all recombinant NH clones present a second hybridization signal over the compression zone (≈ 3.1 Mb), which hybridizes with the *LiSIR2* probe (Fig. 3A) but also

with the *hyg* probe (Fig. 3B). In agreement with the disruption strategy we used, this second hybridization signal revealed by both *LiSIR2* and *hyg* probes might correspond to the *hyg* construct which is in fact maintained extrachromosomally and thus has not been targeted to the WT locus. Increasing agarose concentration from 0.7% to 1.2% led to a shift of this extrachromosomal element to higher molecular weights that can be easily distinguished in PFGE gel stained with ethidium bromide (indicated by arrow in Fig. 3C; lanes NH1, NH2, NH3). Previous reports on the inability of circular DNA to migrate in CHEF analysis, as well as the appearance of smearing when exposition time was increased (not shown), led us to hypothesize that the *hyg* construct was integrated into a circular DNA structure of unknown nature, which was further maintained by the parasite in order to confer drug resistance (Williamson et al., 2002; Beverley, 1988). Characterization of this circular element awaits further investigations, particularly to determine why Southern blot profiles with *Pst*I digestion indicates right genomic integration of the second targeted construct. One possibility is that upstream, and maybe downstream, genomic *LiSIR2* locus has also been rearranged with the *hyg* construct into this extrachromosomal element. This particular event has indeed already been reported in the case of the *trypanothione reductase* (TR) gene disruption in *Leishmania* (Dumas et al., 1997).

Nevertheless, these observations confirm that at least one *LiSIR2* allele is still present in our mutants and strongly support that our failure to obtain null mutants is a direct consequence of the essential role that *LiSIR2* protein plays in parasite survival.

3.3. Episomal complementation is required to obtain null chromosomal mutants

To confirm that *LiSIR2* gene is indispensable to *Leishmania* parasites, we performed episomal rescue experiments by using

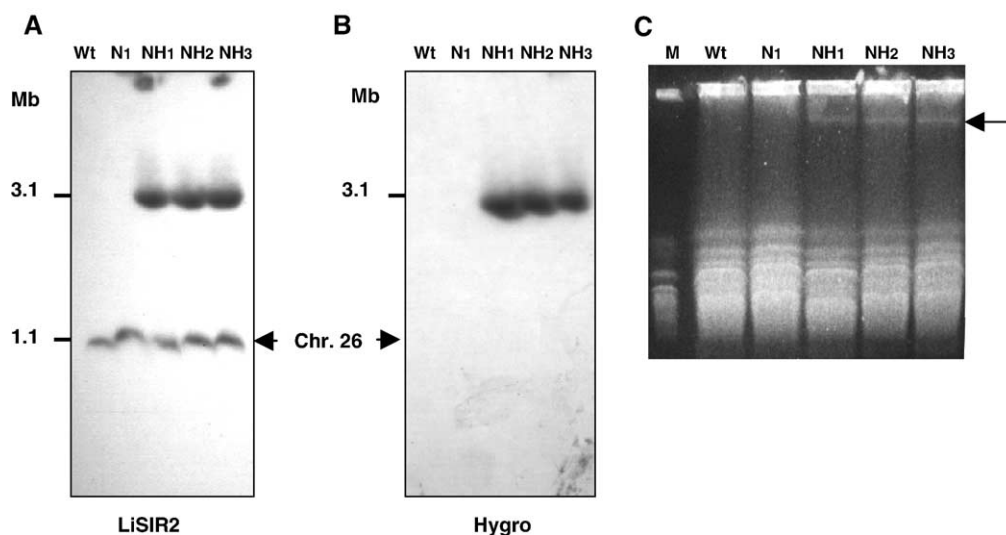


Fig. 3. Karyotype analysis of *Leishmania* clones. 5.10^8 promastigote clones were embedded in agarose plugs and chromosomes were separated by CHEF electrophoresis in 0.7% agarose gel (see Materials and methods for details). Blot transferred chromosomes were hybridized with either the *LiSIR2* (A) or the *hyg* (B) probes. (C) Ethidium bromide staining of a 1.2% agarose gel with same plugs as those in A and B showing an extrachromosomal element in the three NH1, NH2, NH3 clones (marked by arrow). *S. cerevisiae* marker (Bio-rad) is loaded in the first lane (M).

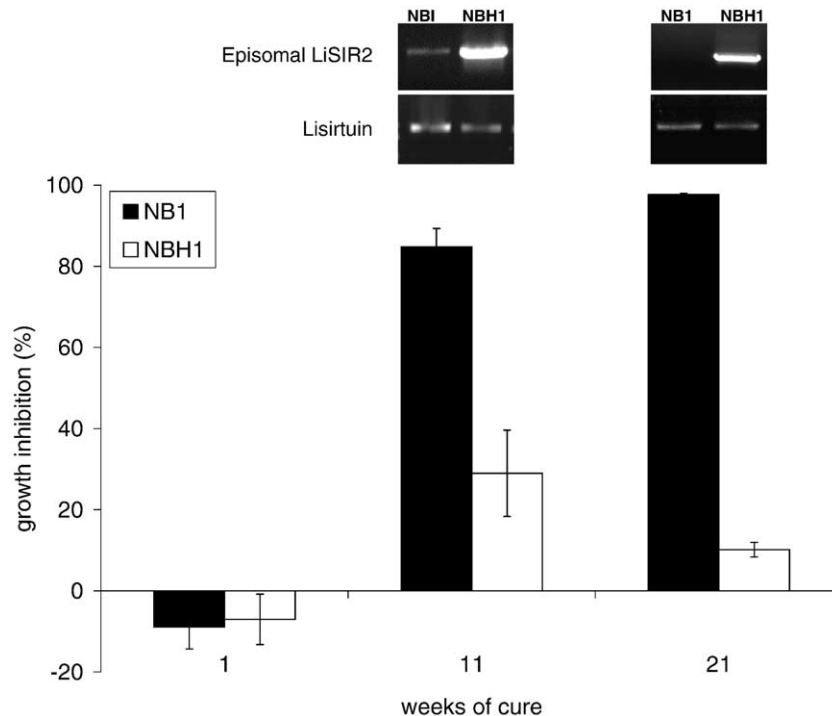


Fig. 4. Plasmid curing experiments. Both NB1 (*LiSIR2/LiSIR2::neo*[pXG-BSD*LiSIR2*]) and NBH1 (*LiSIR2::neo/LiSIR2::hyg*[pXG-BSD*LiSIR2*]) clones were maintained in culture over 21 weeks by weekly subpassages without selection pressure. Plasmid amount was determined at weeks 11 and 21 by both testing parasites sensitivity to blasticidin S (% of growth inhibition in presence of 200 μ g/ml of blasticidin S), and by specifically amplifying *LiSIR2* gene from episome by PCR. *Lisirtuin* gene amplification is used as a control of DNA quantity (see Materials and methods for details).

the pXG-BSD vector exclusively carrying the coding region of *LiSIR2* gene. N1 mutants were transfected with the pXG-BSD*LiSIR2* vector conferring resistance to blasticidin S (NB1 clone: *LiSIR2/LiSIR2::neo*[pXG-BSD*LiSIR2*]). These parasites were then submitted to a second round of transfection with the *hyg* construct. Recombinant “NBH clones” were selected under a triple drug pressure (Geneticin, hygromycin and blasticidin S). Lane NBH1 in Fig. 2F and G demonstrate that, in the presence of a supplementing episome, it was possible to generate null chromosomal mutants of *LiSIR2* (Fig. 2E). These results allowed us to conclude that i) the inability to obtain null *LiSIR2* mutants was not due to a technical bias involving our disruption constructs or a potential alteration of neighboring genes expression; ii) that at least one copy of *LiSIR2* is required to ensure promastigote survival; iii) that the episomal copy of *LiSIR2* gene present on pXG-BSD*LiSIR2* plasmid is able to complement for the loss of protein production associated with complete disruption of *LiSIR2* locus in the promastigote stage of parasite.

As spontaneous plasmid cure could be achieved in *Leishmania* by propagating cells for several weeks in the absence of the selectable drug pressure, we investigated whether or not it could be carried out on NBH1 parasite clone whose survival will rely only on the presence of episomal *LiSIR2* gene copy. The effectiveness of the cure was monitored by using NB1 clone (*LiSIR2/LiSIR2::neo*[pXG-BSD*LiSIR2*]). As NB1 parasites may not require the maintenance of the episomal copy to survive, we hypothesized that vector will be lost over time in the absence of drug pressure. Both NB1 and NBH1 clones were

maintained in culture by weekly subpassages over 21 weeks without blasticidin S selection. The plasmid content was evaluated by testing the degree of promastigotes resistance to blasticidin S and by amplifying episomal *LiSIR2* gene from the pXG-BSD*LiSIR2* plasmid using specific primers. After one week without drug pressure, blasticidin S at 200 μ g/ml was ineffective against both NB1 and NBH1 clones (Fig. 4). However, after 11 weeks, blasticidin S inhibits proliferation of NB1 clone by about 85%, unlike NBH1 parasites which were still virtually insensitive to the drug (Fig. 4A). Additionally, PCR amplification of the episomal *LiSIR2* gene clearly demonstrates that NBH1 clone retained higher amount of pXG-BSD*LiSIR2* plasmid than NB1 clone. Control of DNA quantity is revealed by the similar intensity signal obtained for *Lisirtuin* gene amplification. After 21 weeks of subculture, no episomal *LiSIR2* gene could be detected by PCR in NB1 clones and accordingly, blasticidin S totally inhibit the proliferation of these parasites showing therefore the effectiveness of the cure (Fig. 4). On the contrary, NBH1 clones were still resistant to blasticidin S pressure and showed no significant decrease in episomal *LiSIR2* gene amplification. The absence of plasmid cure in the case of NBH1 clone as compared to NB1 clone strongly support the notion that at least one copy of *LiSIR2* gene is required for *Leishmania* survival. Altogether these results confirm that *LiSIR2* and its encoding gene are essential biomolecules for *Leishmania* survival. Therefore, we investigated whether partial *SIR2* deficiency (single *LiSIR2* knockout clones) might affect the behavior of parasites.

3.4. Involvement of *LiSIR2* protein in axenic amastigote proliferation

Life cycle of *Leishmania* parasite requires two distinct developmental stages (i.e., promastigotes and amastigotes), with different biological properties and nutritional environment (Mauel, 1996). Given that SIR2 proteins have been purposed to be directly regulated by NAD availability and that *Leishmania* SIR2 protein exhibit different cytosolic distribution between promastigote and amastigote forms (Zemzoumi et al., 1998), we have then hypothesized that disruption of *LiSIR2* gene might differentially affect the behavior of the *Leishmania* developmental stages.

Western blot analysis performed on 10^8 promastigotes cells confirms that N1, as well as NBH1 clones, present a substantial decreased in *LiSIR2* expression when compared to WT parental

cell line (Fig. 5A). As expected, the add-back of pXG-BSD*LiSIR2* plasmid into NBH1 clone significantly restores the production of *LiSIR2* protein, yet lower than in *LmSIR2* overexpressing parasites (pSIR2), used as positive control. To further quantify the residual level of *LiSIR2* expression in single knockout clones, flow cytometry analysis was performed using monoclonal antibodies directed against *LmSIR2* hexa-His-tagged fusion protein (mAb IIIG4 and IE10). Disruption of one *LiSIR2* allele reduces the expression of the protein by approximately 50% in promastigotes (Fig. 5B). The various *LiSIR2* mutants were then analyzed for growth characteristics in culture. As shown in Fig. 5C, disruption of one *LiSIR2* allele, as well as overexpressing the gene, does not induce a significant change in promastigotes growth kinetics. Thus even if *LiSIR2* is determinant to *Leishmania* survival, residual level of protein synthesized in N1 clones should be sufficient to ensure normal

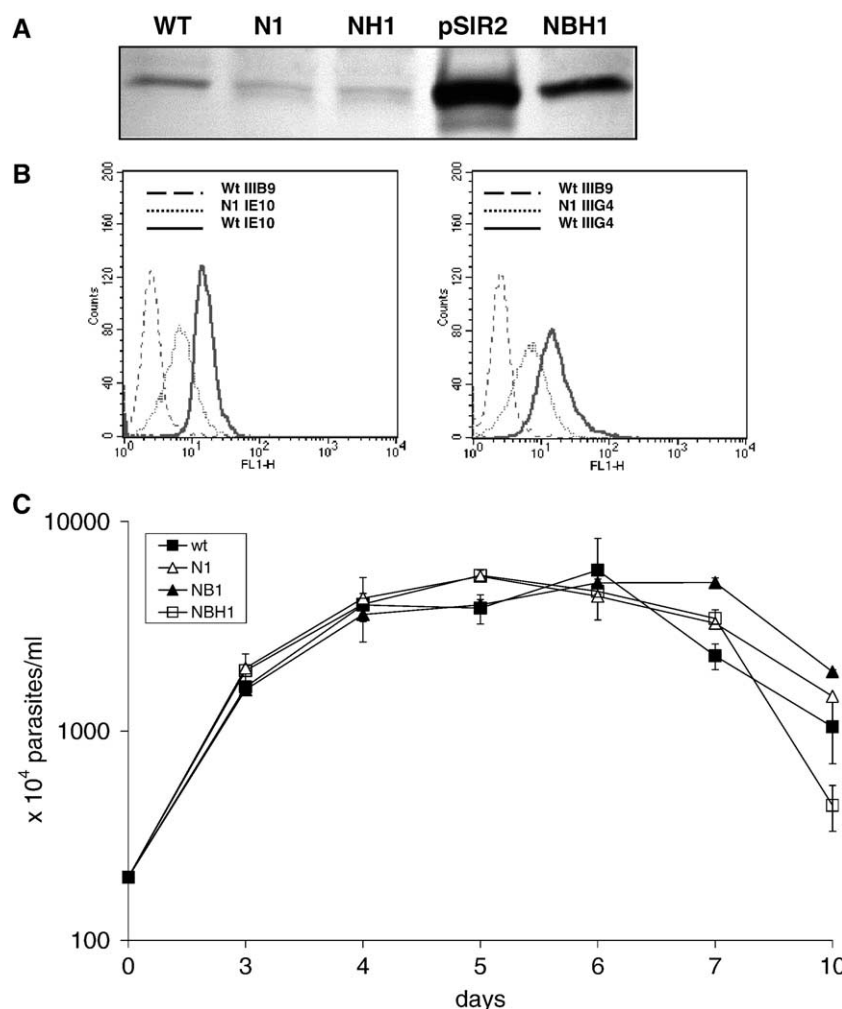


Fig. 5. Characterization of *L. infantum* *LiSIR2* mutant promastigotes. (A) *LiSIR2* protein synthesis examined in equal number (10^8) of viable log phase promastigotes, reacted with polyclonal antibody against *LmSIR2* recombinant protein. WT and overexpressing parasites (pSIR2) were used as controls. N1 or NBH1 clones show a similar reduction in the level of protein synthesis inherent to the disruption of one *LiSIR2* allele. Clone NBH1 derived from plasmid rescue experiment show an increased *LiSIR2* protein expression conferred by pXG-BSD*LiSIR2* episome. (B) FACS analysis confirms the reduction level of *LiSIR2* protein synthesis. WT and N1 clones were reacted with two monoclonal antibodies against *LmSIR2* protein (IIIG4 and IE10) followed by FITC-conjugated goat anti-mouse Ig. IIIB9 mAb which reacted specifically against a *T. cruzi* protein of 24 kDa (Tc24) was used as an internal control (Ouaissi et al., 1992). (C) Growth curve kinetics of promastigote for WT, N1, NB1, and NBH1 clones. Data are the mean of propidium iodide negative cells from two independent growth experiments using as initial inoculum 10^6 parasites/ml.

proliferation of promastigotes. These observations are in agreement with results obtained during plasmid cure. As no differences could be observed for promastigotes, and given that amastigotes express naturally higher amount of LiSIR2 protein (not shown), we monitored growth characteristics of axenic amastigotes. In these conditions, parasites lacking one *LiSIR2* gene allele present a severe proliferation defect (Fig. 6A). This stage specific impairment of parasite proliferation seems to be specific to one *LiSIR2* allele disruption since identical kinetics were systematically observed for five other relative single knockout mutants clones as well as for all other recombinant parasites obtained in this study (NH1, H1, HN1 clones, not shown).

As we generated complemented mutants with pXG-BSD*LiSIR2* plasmid, we then investigated if episomal *LiSIR2* production in single or complemented double knockout clones is able to restore the proliferation defect observed in axenic amastigotes. Unexpectedly, significant growth restoration was observed neither for NB1 nor for NBH1 clones. Furthermore, NBH1 clones, where *LiSIR2* production relies only on pXG-

BSD*LiSIR2*, present an aggravation of the phenotype as they were totally unable to survive in culture (Fig. 6A). These substantial differences between single and double knockout supplemented clones suggest therefore that pXG-BSD*LiSIR2* plasmid cannot ensure proper temporal and/or quantitative expression of the transgene in axenic amastigote, at least during the first step of growth. Indeed, even if complementation is effective in promastigotes (Fig. 5A), as well during differentiation process (not shown), Western blot analysis performed on NB1 amastigotes taken in exponential phase of growth (day 3) indicated that pXG-BSD*LiSIR2* plasmid is not able to restore physiological level of *LiSIR2* protein (Fig. 6B). The absence of defined *Leishmania* promoter sequences and the consequent inability to achieve physiological expression of transgene might results in imprecise or unsuccessful complementation analyses (McKean et al., 2001; Hubel et al., 1997). pXG-BSD*LiSIR2* vector is based on pX series vectors that use regulatory sequences of the *L. major* *DHFR-TS*, a gene which is naturally ten-fold less expressed in *L. mexicana* amastigotes than in promastigotes (Cunningham and Beverley, 2001). Furthermore, failure of

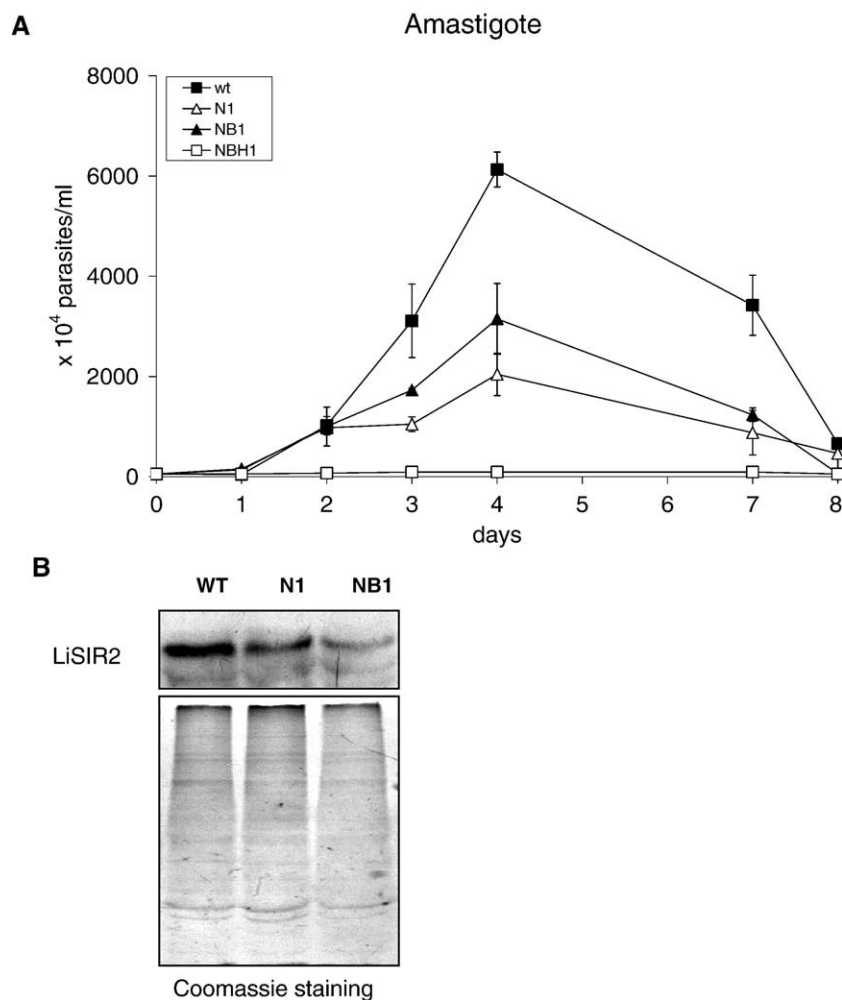


Fig. 6. (A) Growth curve kinetic of axenic amastigotes for WT, N1, NB1 and NBH1 clones. Data are the mean of propidium iodide negative cells from two independent growth experiments using as initial inoculum 2.10^6 parasites/ml. (B) Western blot on carried out on 110 μ g of total protein extract from amastigotes seeded at 2.10^6 parasites/ml and harvested at day three of culture. Lower panel: after protein transfer, the membrane was stained with Coomassie blue to show equivalence of protein loading.

such vectors to ensure expression of transgene in axenic amastigotes has already been reported (Misslitz et al., 2000). Consequently, NBH1 parasite clones are likely to be “conditional mutant” where episomal rescue is effective in promastigotes but not in the amastigote stage of the parasite. Although these results give additional unexpected evidence that the level of *LiSIR2* production is critical for amastigote proliferation in axenic conditions, further studies using plasmid constructs with other 3' and 5' UTR and the *SIR2* gene will be needed to functionally complement the parasite amastigote forms.

3.5. Impairment of mutant parasite clones to replicate in vitro inside macrophages and in vivo in Balb/c mice

The interplay between host cells, particularly macrophages and *Leishmania* parasites, is well characterized. Therefore, we

attempted to determine how the *LiSIR2* mutation affects the parasite ability to infect and replicate within the THP1 human macrophage cell line. In accordance with observations reported in axenic conditions, a marked reduction of the capacity of N1 single mutant amastigotes to proliferate inside macrophages was observed when compared to WT parasites. However, they retain similar capacity than WT to invade macrophages as evidenced by comparable initial parasitic indexes (Fig. 7A).

As the mutant amastigote clones had defective intracellular development in vitro, we were interested in determining whether they could proliferate in a mammalian host. We used a Balb/c mouse model of *L. infantum* infection for the study. Mice were firstly inoculated with 10^8 promastigotes of the parental WT line or the N1 single knockout clone. As shown in Fig. 7B, in animals inoculated with WT cells, parasites burden was $>\text{Log}10 \times 3.5/\text{g}$ of tissue after 2 weeks postinoculation and

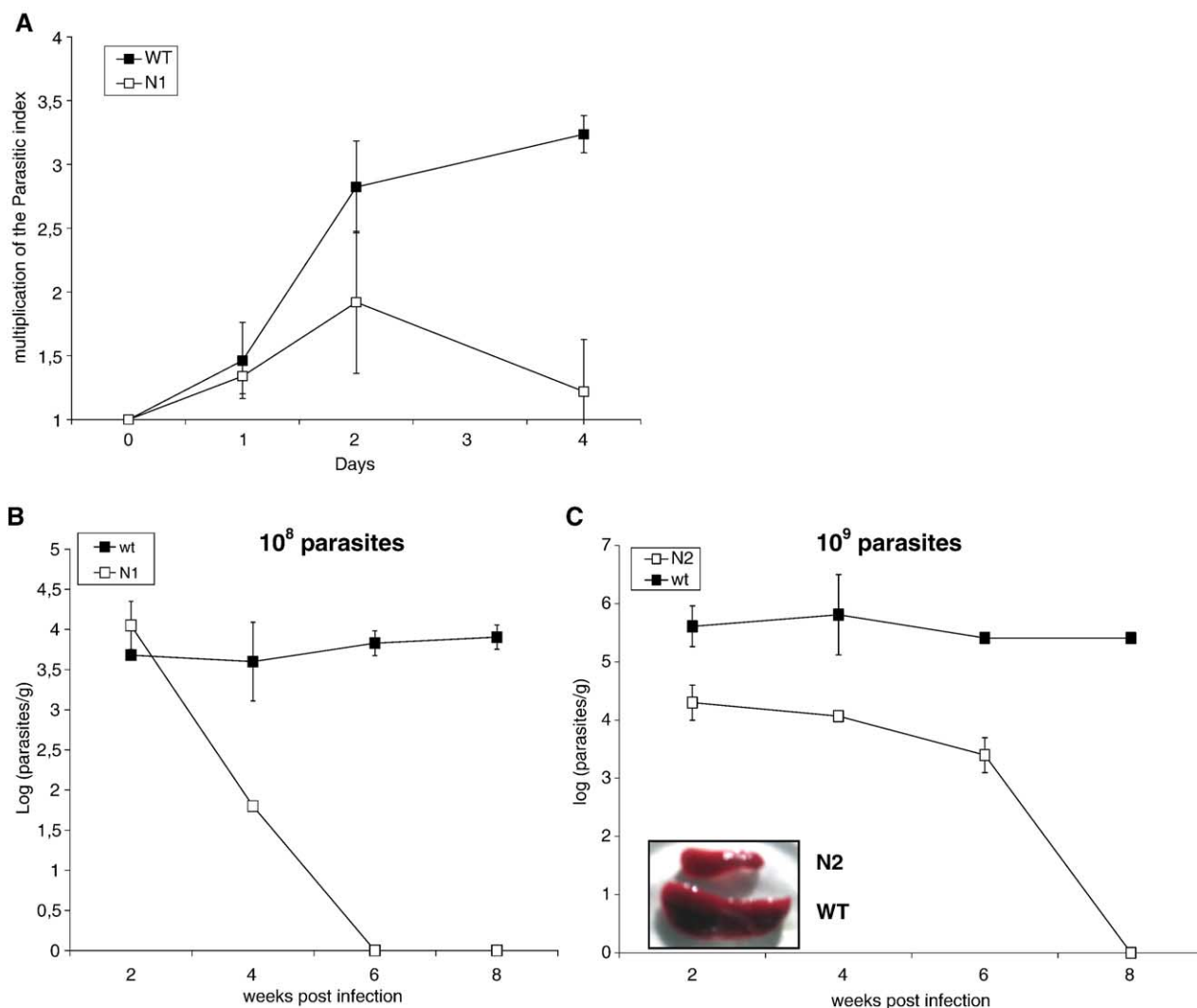


Fig. 7. *LiSIR2* monoallelic disrupted clones show altered infectivity in vitro and in vivo. (A) The in vitro infectivity of WT and N1 mutant parasite clone was conducted using human leukemia monocyte (THP-1 cells)-derived macrophages as host cells following the procedure already described (Serenio et al., 1998). The results represent the mean of triplicate samples and standard deviations are representative of one experiment from two carried out independently. (B) In vivo infectivity of WT and mutant parasite clones. Groups of 12 Balb/c mice were challenged with 10^8 promastigotes of *L. infantum* WT or N1 single mutant clone. Microscopic inspection of serial dilutions of spleen cell homogenates from a group of three mice at each time point, allowed calculating the parasite burden (see Materials and methods). (C) Same experiment conducted with an inoculum of 10^9 promastigotes for both Wt (full square) and N2 clone (open square). Insert: picture showing comparative splenomegaly of mice infected with Wt or N2 clone removed 9 weeks after parasites inoculation.

remained high over the study period (up to 8 weeks post infection). In contrast, although N1 mutant clones were infective, parasites being detected after 2 weeks of infection, a progressive reduction in the parasite burden is observed. No parasites can be detected in the tissue samples after 6 weeks, suggesting that they failed to establish an infection. Increasing inoculums to 10^9 promastigotes led to an increased parasite burden in the first weeks of infection for both WT and N2 parasites. Nevertheless, a similar decline is observed for N2 clones 6 weeks after infection, and no parasites could be detected 8 weeks post infection (Fig. 7C). Interestingly, infection of Balb/c mice with single knockout N2 clone resulted in a strong reduction of splenomegaly as judged by examination of the organs (Insert in Fig. 7C). These results agree with the in vitro observation using macrophages as a host cell, and also suggest the clearance of the parasites by the mammalian host.

4. Discussion

Targeted disruption of the *Leishmania* SIR2 cytosolic encoding gene was performed to determine biological significance of this protein for the parasite. In contrast to the common expectation, we provide strong arguments that this gene is essential for parasite survival. Attempts to generate mutants in which both copies of *LiSIR2* were disrupted by specific integration of a gene-targeting construct invariably failed. One allele of *LiSIR2* can be deleted with either a *neo* or *hyg* construct but attempts to delete the second allele resulted in the integration of the targeting construct into an episomal element distinguishable in PFGE analysis. The mechanism by which this event occurs upon gene targeting is unknown at present, however such phenomenon is generally considered as indirect evidence that the gene being targeted for disruption is essential. Complementation with an episome exclusively carrying the coding region of *LiSIR2* gene allowed us to disrupt both chromosomal *LiSIR2* alleles confirming therefore that the inability to generate null chromosomal mutants was related to the fact that this gene possesses essential function for parasite survival. Further indirect evidence comes from observation that pXG-BSD*LiSIR2* plasmid was lost in single knockout parasites but was maintained in double knockout clones, even after more than 5 months of culture in the absence of drug pressure demonstrating therefore the essential role of SIR2 gene for parasite survival. These observations are in agreement with our previous finding showing that overexpression of SIR2 promotes survival of *Leishmania* amastigotes (Vergnes et al., 2002). Phenotypic analysis in both developmental stages suggests a stage specific requirement and/or activity of this protein. However, further studies using other plasmid constructs containing regulatory 3' and 5' UTRs and *SIR2* gene are needed to attempt to restore the culture growth and virulence of SIR2 double knock out amastigote forms.

To our knowledge, this is the first study reporting that a *SIR2* related gene is essential to an organism. This presumes that *LiSIR2* and possibly the other SIR2 related proteins of *Leishmania* might be involved into unrelated biological pathway unique to this parasite organism. A recent study

focusing on a nuclear SIR2 related protein from the protozoan parasite *T. brucei* (TbSIR2RP1) reported an exclusive ability of this protein to combine both NAD-dependant deacetylase and ADP-ribosyltransferase activity on histones (Garcia-Salcedo et al., 2003). Although this activity has not yet been tested for *Leishmania* protein, it may suggest that protozoan SIR2 proteins might share exclusive biological properties which can be determinant for virulence or survival of these parasitic organisms. Recent data support the idea that SIR2 proteins were firstly arisen in primordial eukaryotes to help them cope with adverse conditions by acting as metabolic or redox sensor (Lamming et al., 2004).

In our model system, we have previously shown that cytosolic SIR2 protein act on parasite survival under stress conditions (Vergnes et al., 2002). Our present finding showing the implication of *LiSIR2* protein in amastigote cell cycle progression suggests that this protein may be involved in parasite adaptation by both controlling multiplication rate and survival capacity of amastigotes within host cells according to environmental conditions. Therefore, the identification of the substrate(s) targeted by the *Leishmania* cytoplasmic SIR2 during the life cycle of the parasite will shed light on the putative biochemical pathway(s) involved. Two major therapeutic perspectives emerge from this study. Firstly, genetically modified parasites are considered as a powerful alternative to develop new generation of vaccine against leishmaniasis (Titus et al., 1995). The inability of *LiSIR2* single knockouts amastigotes to proliferate inside macrophages as well as their progressive elimination in Balb/c mice suggest that *L. infantum* live attenuated *LiSIR2* mutants could be useful for vaccine development strategies in the future. Secondly, given that deletion of one allele of *LiSIR2* locus is sufficient to dramatically affect amastigote proliferation, and that the sequence homology with host SIR2 proteins is enough divergent, this protein might be considered as a new interesting target to develop specific chemotherapeutic inhibitors against parasite. Investigations along these lines of research are in progress in our laboratory.

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2.3 Structure function analysis of *Leishmania* sirtuin: an ensemble of *in silico* and biochemical studies

Novel anti-leishmanial target LmSir2 has few subtle but prudent structural differences in ligand binding and catalytic domain as compared to its human counterpart. *In silico* screening and validation followed by *in vitro* deacetylation and cell killing assays described herein give a proof of concept for development of strategies exploiting such minor differences for screening libraries of small molecules to identify selective inhibitors.

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Structure Function Analysis of *Leishmania* Sirtuin: An Ensemble of *In Silico* and Biochemical Studies

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Novel anti-leishmanial target LmSir2 has few subtle but prudent structural differences in ligand binding and catalytic domain as compared to its human counterpart. *In silico* screening and validation followed by *in vitro* deacetylation and cell killing assays described herein give a proof of concept for development of strategies exploiting such minor differences for screening libraries of small molecules to identify selective inhibitors.

Key words: leishmaniasis, nicotinamide and virtual screening, Sir2

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Spread of leishmaniasis which is an opportunistic infection in HIV patients (1), pregnancy and in organ transplant (2), has increased in recent years due to globalization and increased travel (3). Novel biochemical targets such as sirtuins are being explored for drug development (4–6) to tackle the problems of the currently available treatments. The sirtuin family of proteins, also called the Silent Information Regulator 2 or Sir2 family, consists of broadly conserved NAD⁺-dependent deacetylases that are implicated in diverse biological processes, such as transcriptional silencing by deacetylating histones, regulation of apoptosis by deacetylating p53, metabolism and longevity by deacetylating yet to be uncovered substrates. Sir2 proteins are physiologically regulated in part by the cellular concentrations of a non-competitive inhibitor, nicotinamide which reacts with a reaction intermediate *via* a base-exchange reaction

to reform NAD⁺ at the expense of deacetylation (7). Literature elucidates insights into the mechanism of Sir2 inhibition by nicotinamide and has important implications in the development of Sir2 specific inhibitors (7,8). Since Sir2 is also present in human, screening of inhibitors for the purpose of drug design needs to consider selectivity for the leishmanial target. In this communication we report *in silico* target-based screening of 2×10^5 NCI compounds followed by *in vitro* enzymatic and cell-based assays. Our findings provide a proof of concept that the subtle structural differences in ligand binding and catalytic domains of *Leishmania* and human Sir2 (LmSir2 and hSir2) can be exploited for screening more selective LmSir2 inhibitors.

Materials and Methods

Computational procedure

The National Cancer Institute 3D database (NCI-2000)^a containing approximately 2×10^5 compounds was screened using a nicotinamide fingerprint. The screened molecules were then docked into the crystal structure of hSir2 available under PDB code 1J8F and a recently published homology model of LmSir2 (10) to identify putative selective binders. Docking was performed using FlexX module in SYBYL 6.9 software^b. At least 30 docking conformations were calculated for all the ligands. The docking methodology was confirmed by a two-step validation process as described in Results and Discussion. The same protocol was then used for docking the molecules selected from fingerprint-based screening of NCI database to *Leishmania* and human sirtuin.

Compounds for experimental analysis

All compounds except nicotinamide (Sigma, St Louis, MO, USA) were provided from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute. The stock solutions (0.5 M) were prepared in dimethyl sulfoxide and stored at 4 °C. Working solutions were freshly prepared using culture medium till the desired final concentration for the different assays.

NAD⁺-dependent deacetylase assay

To evaluate the effect of the compounds on *Leishmania* NAD⁺-dependent deacetylase activity, extracts from mutant parasites

carrying extra copies of the *Leishmania major* *SIR2RP1* were used in the commercially available Cyclex Sir2 assay kit (Cyclex Co., Ltd., Nagano, Japan). Briefly, 1×10^7 mutant parasites were washed twice with saline phosphate buffer at pH 7.4 (PBS) and lysed in a buffer containing: 100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5 mM TSA pH 8.8, and placed on ice for 30 min. The cells were then centrifuged at 10,600 *g* for 20 min at 4 °C and the supernatant was collected. The effect of the compounds on deacetylase activities was measured in the presence or absence of 200 μ M NAD to discriminate between the NAD⁺-dependent and independent activity. The effect of the compounds was evaluated according to the manufacturer instructions for Cyclex Sir2 kit.

Parasites and growth inhibition assays

Leishmania infantum (clone MHOM/MA671TMAP263) axenic amastigotes expressing luciferase (5) were cultured at 37 °C, and 5% CO₂ in a cell-free medium called MAA/20 (medium for axenic amastigotes growth). MAA/20 consisted of modified medium 199 (Gibco) with Hank's Balanced Salt Solution supplemented with 0.5% trypto-casein (Oxoid, Hampshire, UK), 15 mM D-glucose (Sigma), 5 mM glutamine (Gibco), 4 mM NaHCO₃, 0.023 mM bovine hemin (Fluka) and 25 mM HEPES (Gibco) to a final pH of 6.5 and supplemented with 20% heat inactivated fetal bovine serum (Gibco, Invitrogen Corp. CA, USA). For the growth inhibition assays, 1×10^6 luciferase expressing axenic amastigotes/ml were seeded in a flat bottom 96-well plate and incubated with the different compounds at 37 °C with 5% CO₂ for 72 h. At the end, the parasites viability was evaluated by the luciferase assay. The percentage of growth for each drug concentration was calculated as following (RLU with drug/RLU without drug) *100) where RLU is Relative Luciferase Unit.

Luciferase assay

Luciferase assay was performed as described by Roy *et al.* (9) results expressed as RLU.

Statistical analysis

The data were analysed using Student's *t*-test.

Results and Discussion

A detailed computational analysis exploring the differences in the LmSir2 and hSir2 was recently reported (10), opening the possibility of identifying selective LmSir2 inhibitors. As illustrated in Figure 1, a novel *in silico* screening strategy based on the subtle differences in the ligand binding and catalytic domains of the two proteins, was implemented to identify selective inhibitors of LmSir2 over hSir2. The virtual screening procedure consisted of fingerprint-based search and docking.

The 3D structural NCI database was imported into the MOE (Molecular Operating Environment) package^c for similarity search. Three point pharmacophore fingerprints called the pharmacophore atom triangle fingerprints were generated for all molecules in database. Pharmacophoric fingerprint of nicotinamide with a Tan-

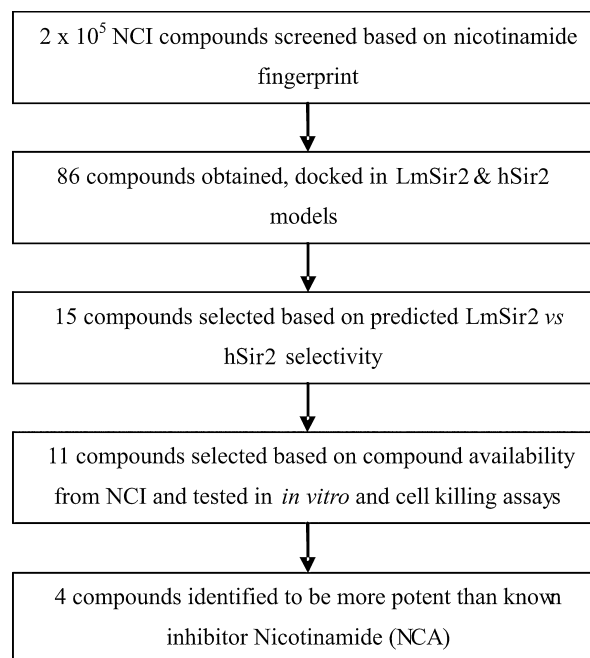


Figure 1: Flow diagram of screening protocol used in the study.

imoto coefficient cutoff of 60% was used to query the database. This yielded 86 molecules, which were then loaded into SYBYL6.9 with the purpose of carrying out a comparative docking analysis against LmSir2 and hSir2. The docking strategy to be employed for the analysis was validated in two steps. In the first step the bound conformation of nicotinamide (NCA) was extracted from the crystal structure of Sir2Af2 (PDB code 1YC2) and redocked into its binding site. The docked NCA showed essentially the same conformation as the bound ligand with a RMSD value of 0.256 Å calculated using fit atom method in SYBYL 6.9. As expected from literature, the docked NCA was anchored by the carboxamide group, leaving the pyridine ring free to pivot on its only rotatable bond inside the pocket (7,8). The second validation step consisted of docking the NCA and nicotinic acid (NAC) to modelled LmSir2 and comparing the results against the reported biological activities (4). NCA which gave a better docking score of 10.9 kcal/mol is known to be more potent (IC₅₀ 5.5 ± 0.5 mM) than NAC which gave a poorer docking score of 8.6 kcal/mol and is also less potent (IC₅₀ 12.7 ± 2 mM). In accordance with the reported literature, NCA docked to LmSir2 showed interactions with the highly conserved 'TONID' motif (7). The carbonyl oxygen of carboxamide group made hydrogen bonding interactions with hydrogen attached to back bone nitrogen atom of Ile126 and the hydroxyl oxygen of Asn125 hydrogen bonded with amide hydrogen of NCA (8) (Figure 2B).

The validated docking strategy was then employed to dock the 86 molecules identified from the fingerprint search into LmSir2 and hSir2. Most of the molecules docked into both the active sites comparably. However, a few molecules demonstrated better docking scores to either LmSir2 or hSir2 (Figure 3; Table 1), indicating that the structural differences of the active site residues were playing a

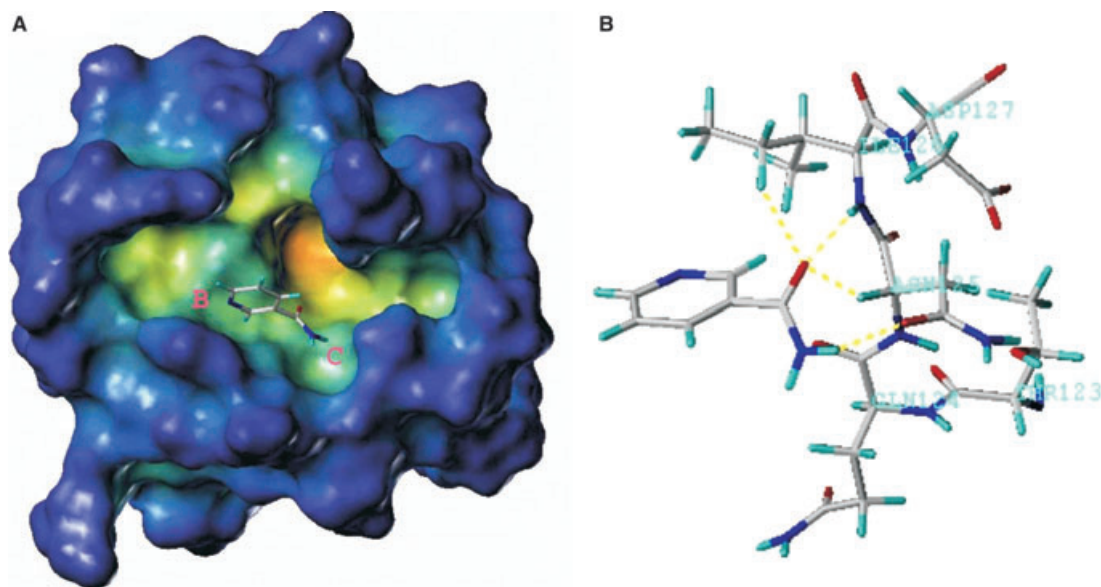


Figure 2: LmSir2 model. (A) MEPS surface showing docking of nicotinamide. (B) Interaction of nicotinamide with active site residues.

discriminatory role in protein–ligand interactions. Docking score-based classification divided the compounds into following three groups: group one containing compounds which docked selectively in LmSir2, group two containing compounds which docked selectively into hSir2 and a third group containing compounds with similar docking score for both the proteins. A set of 15 compounds was selected from the three groups (five from each group) and requested from NCI for biological testing; 11 of the 15 compounds were available.

To test the *in vitro* inhibitory activity of the compounds, NAD⁺-dependent deacetylase assays were performed using extracts from recombinant *L. infantum* parasites carrying extra copies of LmSIR2RP1, gene encoding leishmanial sirtuin. The obtained results

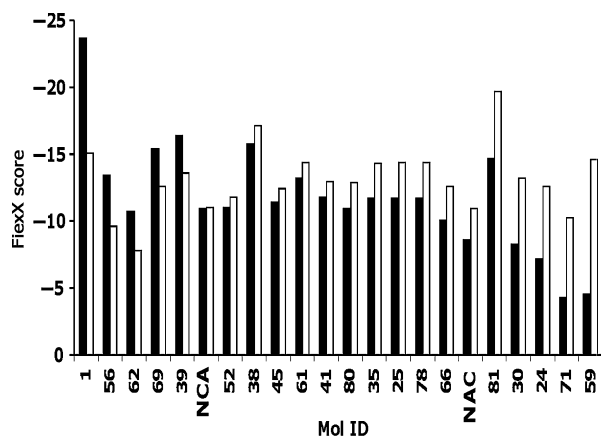


Figure 3: Virtual screening result of representative molecules with varying selectivity for *Leishmania* (black) and human (white) sirtuin are shown in the figure.

showed that compound **56** was comparatively more active as an inhibitor of parasite deacetylase activity in overexpressed extract at 2.5 mM concentration, whereas compounds **1** and **75** showed only 20% inhibition, as compared to NCA (Figure 4A). Surprisingly, compound **42** tended to have an enhancing effect on deacetylase activ-

Table 1: Docking scores of and amastigote growth inhibition by the four active compounds and NCA

Mol ID	Structure	FlexX Score		
		LmSir2	hSir2	IC ₅₀ (mM)
1		-23.7	-15.1	0.49 ± 0.009
42		-16.1	-16.9	0.28 ± 0.036
56		-13.4	-9.6	1.49 ± 0.021
75		-4.2	-8.6	0.82 ± 0.079
NCA		-10.9	-11.0	5.5 ± 0.05

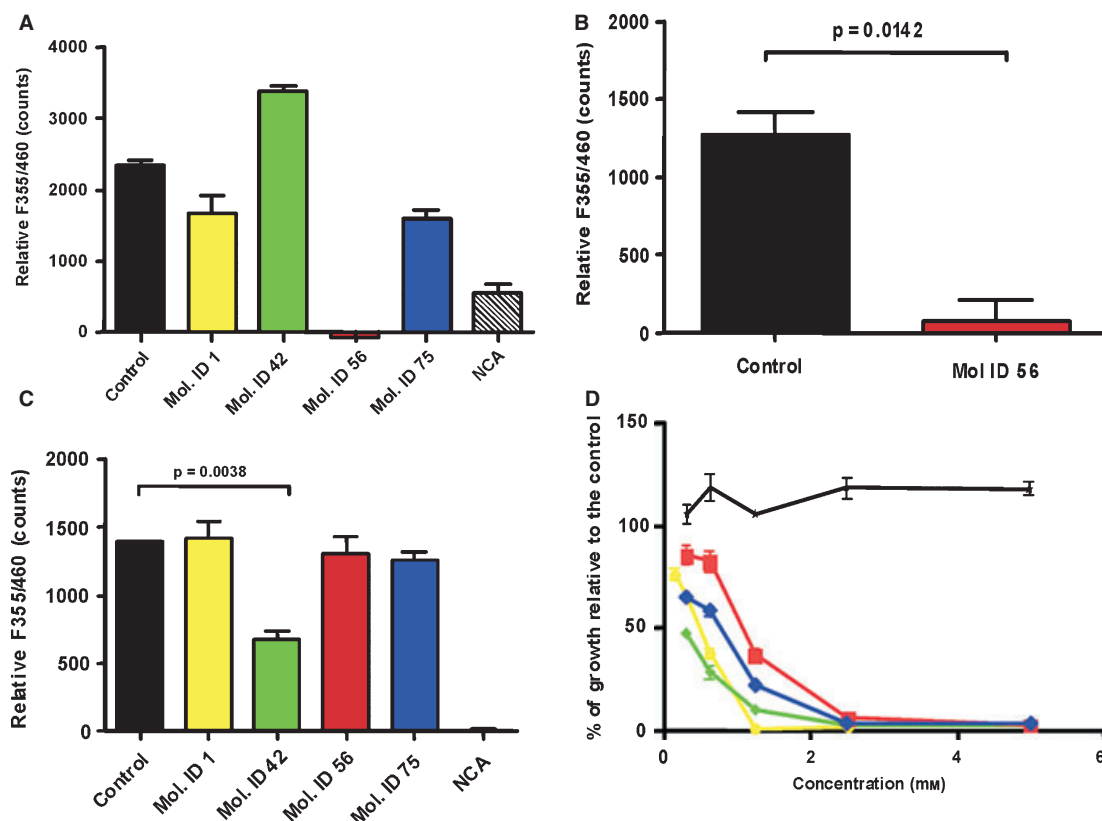


Figure 4: (A) LmSir2 inhibition by NSC compounds **1**, yellow; **42**, green; **56**, red and **75**, blue at 2.5 mM concentration obtained from an average of three independent experiments. (B) Effect of compound **56** on the NAD⁺-dependent deacetylase activity of axenic amastigotes extract that overexpress LmSir2RP1 obtained from an average of seven independent experiments. (C) Effect of NCI compounds on the NAD⁺-dependent deacetylase activity of the recombinant human sirtuin. (D) *Leishmania infantum* axenic amastigotes growth inhibition by NSC compounds **1**, yellow; **42**, green; **56**, red and **75**, blue at 2.5 mM concentration after 72 h of incubation.

ity at the same concentration. Studies were then performed on hSir2 with the purpose of comparing against the effects observed in LmSir2. As shown in Figure 4C, compounds **1**, **56** and **75** showed no inhibitory activity at 2.5 mM concentration whereas compound **42** was able to inhibit approximately 50% of the hSir2 deacetylase activity as compared to NCA.

In vivo activity of compounds was tested using parasite growth inhibition assay on *L. infantum* axenic amastigotes expressing luciferase, cultured at 37 °C in presence and absence of 2.5 mM of test compounds. All four compounds could inhibit growth of axenic amastigotes after 72 h of incubation. To calculate IC₅₀ values of growth inhibition, axenic amastigotes were incubated with the different concentration of compounds for 72 h and parasite viability was evaluated by the luciferase assay. The percentage of growth for each compound concentration was calculated (Figure 4D) and IC₅₀ values were determined. This experiment demonstrated that all four compounds were efficient in inhibiting the amastigote growth when compared to nicotinamide (Table 1), even though only compound **56** might be acting *via* LmSir2 inhibition (Figure 4B). The mechanism of action of compounds **1**, **42** and **75** remains unclear.

To gain further insight into the selective inhibition of leishmanial protein as compared to human counterpart by compound **56** a detailed analysis of the docked compound into LmSir2 and hSir2 was performed (Figure 5). Docking results for LmSir2 indicate that like NCA, compound **56** interacts with 'TQNID' motif making hydrogen bonding interactions with hydroxyl oxygen of Asn125 and back bone nitrogen atom of Ile126. Additionally, compound **56** shows hydrogen bonding of fluorine with active site residue Gly39 and π -stacking interactions with Phe51 (Figure 5A). Whereas in hSir2, compound **56** shows hydrogen bonding interactions only with the highly conserved residues His187 and Gln 267 (Figure 5B). Thus, the selective activity of this compound might be attributed to additional hydrogen bonding and π -stacking interactions with the leishmanial protein. Moreover the surface around the C-pocket in active site would also have a variable impact on binding and orientation of the ligands (Figure 2A).

Furthermore, drug likeness of these compounds was confirmed to analyse their lead potentiality. Table 2 suggests that all four compounds are well within the acceptable limits of Lipinski's rule of five. Even though these hits show promising results for activity and selectivity as compared to NCA, and are also drug like, their overall

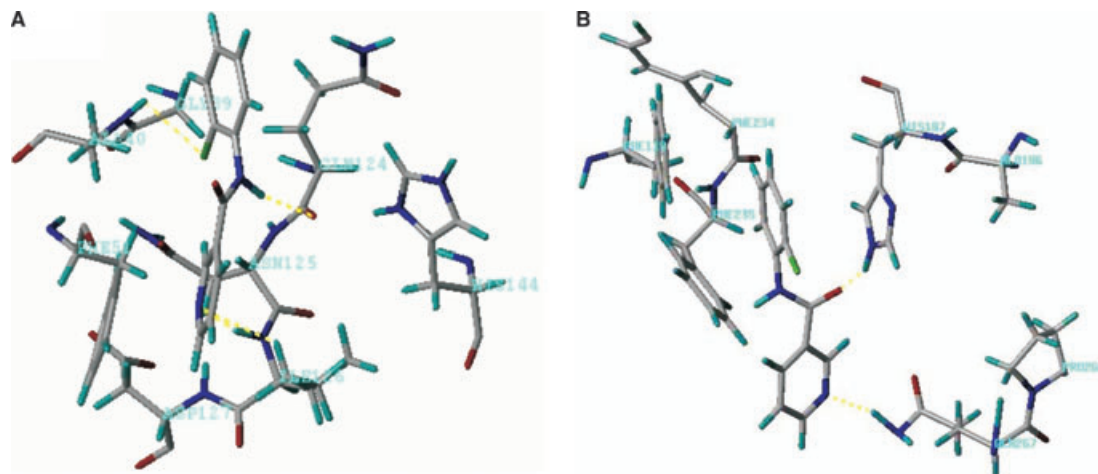


Figure 5: Compound **56** selectively docked in *Leishmania* sirtuin (A) as compared to human sirtuin (B).

Table 2: The drug-likeness profile of compounds effective in killing leishmanial axenic amastigotes

Mol ID	MW	Rot Bond	mi-LogP	TPSA	RO5 violations
1	152.157	1	−1.144	94.036	0
42	228.251	4	1.513	65.22	0
56	216.215	2	1.707	41.99	0
75	170.237	3	0.305	55.986	0

MW, Molecular Weight; Rot bond, Rota table bonds; miLogP, molinspiration LogP; TPSA, Total Polar Surface Area and RO5 violations, Rule of Five violations.

activity profile is too low for them to be directly considered as lead for selective LmSir2 inhibitor design.

Conclusion

An ensemble of *in silico* and biochemical studies was imbibed to implement a novel screening strategy based on subtle differences in ligand and catalytic binding site of two homologous proteins. Docking studies and biological assay results in corroboration with the drug-like properties revealed that it is possible to identify selective inhibitors of LmSir2 over the corresponding human enzyme. Even though we did not succeed in identifying a truly potent and selective lead compound, the presented strategy was effective in identifying compound **56** which can be used for lead designing. The reported screening methodology was employed on Sirtuin enzyme, however, it can have wide spread applicability extending to other targets as well. From the present study it could also be concluded that the anti-leishmanial activity of compounds **1**, **42** and **75** was not because of the inhibition of LmSir2. Physiological relevance of augmentation of sirtuin activity and cell death needs to be explored further.

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Notes

^ahttp://dtp.nci.nih.gov/docs/3d_database/dis3d.html.

^bSYBYL Software Package, version 7.1. St Louis, USA: Tripos Associates Inc.

^cMOE Software Package, version 06. Montreal, Canada: Chemical Computing Groups, Inc.

2.4 The *Leishmania infantum* cytosolic SIR2 related protein 1 (LiSIR2RP1) is an NAD⁺-dependent deacetylase and ADP-ribosyltransferase

Proteins of the SIR2 (Silent Information Regulator 2) family are characterized by a conserved catalytic domain that exerts unique NAD⁺-dependent deacetylase activity on histones and various other cellular substrates. Previous reports from us have identified a *Leishmania infantum* gene encoding a cytosolic protein termed LiSIR2RP1 (*Leishmania infantum* SIR2-related protein 1) that belongs to the SIR2 family. Targeted disruption of one LiSIR2RP1 gene allele led to decreased amastigote virulence, *in vitro* as well as *in vivo*. In the present study, attempts were made for the first time to explore and characterize the enzymatic functions of LiSIR2RP1. The LiSIR2RP1 exhibited robust NAD⁺-dependent deacetylase and ADP-ribosyltransferase activities. Moreover, LiSIR2RP1 is capable of deacetylating tubulin, either in dimers or, when present, in taxol-stabilized microtubules or in promastigote and amastigote extracts. Furthermore, the immunostaining of parasites revealed a partial co-localization of α -tubulin and LiSIR2RP1 with punctate labelling, seen on the periphery of both promastigote and amastigote stages. Isolated parasite cytoskeleton reacted with antibodies showed that part of LiSIR2RP1 is associated to the cytoskeleton network of both promastigote and amastigote forms. Moreover, the Western blot analysis of the soluble and insoluble fractions of the detergent of promastigote and amastigote forms revealed the presence of α -tubulin in the insoluble fraction, and the LiSIR2RP1 distributed in both soluble and insoluble fractions of promastigotes as well as amastigotes. Collectively, the results of the present study demonstrate that LiSIR2RP1 is an NAD⁺-dependent deacetylase that also exerts an ADP-ribosyltransferase activity. The fact that tubulin could be among the targets of LiSIR2RP1 may have significant implications during the remodelling of the morphology of the parasite and its interaction with the host cell.

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The *Leishmania infantum* cytosolic SIR2-related protein 1 (LiSIR2RP1) is an NAD⁺-dependent deacetylase and ADP-ribosyltransferase

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Proteins of the SIR2 (Silent Information Regulator 2) family are characterized by a conserved catalytic domain that exerts unique NAD⁺-dependent deacetylase activity on histones and various other cellular substrates. Previous reports from us have identified a *Leishmania infantum* gene encoding a cytosolic protein termed LiSIR2RP1 (*Leishmania infantum* SIR2-related protein 1) that belongs to the SIR2 family. Targeted disruption of one LiSIR2RP1 gene allele led to decreased amastigote virulence, *in vitro* as well as *in vivo*. In the present study, attempts were made for the first time to explore and characterize the enzymatic functions of LiSIR2RP1. The LiSIR2RP1 exhibited robust NAD⁺-dependent deacetylase and ADP-ribosyltransferase activities. Moreover, LiSIR2RP1 is capable of deacetylating tubulin, either in dimers or, when present, in taxol-stabilized microtubules or in promastigote and amastigote extracts. Furthermore, the immunostaining of parasites revealed a partial co-localization of α -tubulin and LiSIR2RP1 with punctate labelling, seen on the periphery of both promastigote and amastigote stages. Isolated parasite cyto-

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Key words: ADP-ribosyltransferase, cytoskeleton, *Leishmania infantum* SIR2-related protein 1 (LiSIR2RP1), NAD⁺-dependent deacetylase, parasite, α -tubulin.

INTRODUCTION

The yeast SIR2 (Silent Information Regulator 2) protein is the founding member of a large conserved family of proteins that are present in many organisms ranging from bacteria to humans [1]. This protein is an NAD⁺-dependent HDAC (histone deacetylase) that modulates the chromatin structure, and silences the transcription at the level of the silent mating type loci, telomeres and ribosomal DNA [2–4]. In addition to epigenetic silencing, the yeast SIR2 also has a role in DNA repair, recombination and DNA replication [5]. The first evidence that the SIR2 proteins could be involved in functions other than transcriptional silencing came from the presence of the *Leishmania major* SIR2-related protein in cytoplasmic granules [6]. Seven SIR2 genes were identified in humans, and the proteins, classified as sirtuins (SIRT1–7), present several subcellular localizations [7,8]. However, not all of the sirtuin family members seem to be active deacetylases [9,10]. SIRT1 is localized in the nucleus and deacetylates the histones H3 and H4, p53, TAF168 and PCAF [p300/CREB (cAMP-response-element-binding protein)-binding

protein-associated factor]/MyoD [4,11–14]. Despite the cytosolic localization of SIRT2 and its ability to deacetylate α -tubulin, this protein shuttles the nucleus during G₂/M transition and thus plays a role in the regulation of microtubule dynamics and cell-cycle progression [15–17]. SIRT3 was found in the mitochondria controlling the activity of the acetyl-CoA synthetase 2 [18]. Histone and non-histone substrates are deacetylated by sirtuins in the presence of NAD⁺ and lead to the production of the deacetylated protein, nicotinamide and OAADPr (O-acetyl-ADP-ribose) [19,20]. Several questions have been raised on understanding the NAD⁺ dependency of the sirtuin, since there is no energetic reason that favours the coupling. Among the possibilities is the connection between the proteins deacetylation and the metabolic status of the cell or the formation of a second messenger, OAADPr [20].

Some members of the SIR2 family, such as the yeast SIR2, the human SIRT2, the mouse SIRT6, the *Trypanosoma brucei* SIR2RP1 and the *Plasmodium falciparum* SIR2, exhibit ADP-ribosyltransferase activity [7,21–24]. This kind of enzymatic activity was for the first time attributed to this family of proteins

Q6 Abbreviations used: APC, ?????; DAPI, 4',6-diamidino-2-phenylindole; FBS, foetal calf serum; HDAC, histone deacetylase; LicTXNPx, *Leishmania infantum* cytosolic peroxiredoxin; OAADPr, O-acetyl-ADP-ribose; SIR2, Silent Information Regulator 2; LiSIR2RP1, *Leishmania infantum* SIR2-related protein 1; LmSIR2, *Leishmania major* SIR2; SIRT, sirtuin; rLiSIR2RP1, recombinant LiSIR2RP1; rSIRT1, recombinant SIRT1; TCA, trichloroacetic acid; TSA, trichostatin A; wt, wild-type.

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due to the ability of the *Salmonella typhimurium* SIR2 homologue, Cob B, to compensate for the loss of Cob T in cobalamin biosynthesis [25].

Additionally, sirtuins, and particularly the one associated with caloric restriction regimens in several organisms such as *Caenorhabditis elegans*, yeast and fly might also be involved in longevity [26–28].

The agent of leishmaniasis, the protozoan parasite *Leishmania*, is responsible for serious diseases characterized by diverse clinical manifestations varying from localized ulcerative skin lesions and destructive mucosal inflammation to the disseminated visceral infection also known as Kala azar. The visceral leishmaniasis caused by *L. infantum* is a serious problem throughout the Mediterranean basin, where dogs are the most important reservoirs of the parasite. A vertebrate host becomes infected by *Leishmania* after the bite of a sandfly (*Phlebotomus* and *Lutzomyia* spp) during a blood meal, through the inoculation of infective flagellated promastigotes that invade or are phagocytosed by local or recruited host cells. In the phagolysosomes, the promastigotes will differentiate into non-flagellated amastigotes that multiply and are able to infect other adjacent or distant macrophages. The classical treatment is currently unsatisfactory due to side effects, the appearance of resistances and the need for increased efficacy in immunosuppressed patients, especially due to HIV co-infection. Over last few years, our group has been interested in the *Leishmania* SIR2 proteins due to the role of LiSIR2RP1 (*L. infantum* SIR2-related protein 1) in the survival and virulence of the parasite [29,30].

Although few reports have disclosed the SIR2 protein function in the protozoan parasites, such as *P. falciparum*, whose PfSIR2, localized in the nucleus, is involved in *var* gene silencing through heterochromatin formation [31], and *T. brucei*, which has a chromosome-associated NAD⁺-dependent deacetylase and ADP-ribosyltransferase that confers resistance to DNA damage [22], little is known about the *Leishmania* cytosolic LiSIR2RP1 biological properties. In the present study, we provide molecular and biochemical evidence supporting the notion that LiSIR2RP1 is an NAD⁺-dependent deacetylase enzyme that may also express ADP-ribosyltransferase activity. In addition, we show that the protein deacetylates α -tubulin and is partially associated with the microtubule network. Further studies on LiSIR2RP1 may provide new insights into the relationships among cytoskeleton dynamics, stage transition and parasite–host cell interaction.

EXPERIMENTAL

Parasites

The *L. infantum* (clone MHOM/MA/67/ITMAP-263) wt (wild-type) promastigotes were grown at 27°C in RPMI 1640 medium (Cambrex) supplemented with 10% heat-inactivated FBS (foetal bovine serum; Cambrex), 2 mM L-glutamine (Cambrex), 20 mM Hepes (Cambrex), 100 units/ml penicillin (Cambrex) and 100 µg/ml streptomycin (Cambrex). *L. infantum* axenic amastigote forms were grown at 37°C with 5% CO₂ in a cell-free medium called MAA/20 (medium for axenic amastigotes). MAA/20 consisted of modified medium 199 (Gibco) with Hank's balanced salt solution supplemented with 0.5% trypto-casein (Oxoid), 15 mM D-glucose (Sigma), 5 mM glutamine (Cambrex), 4 mM NaHCO₃ (Sigma), 0.023 mM bovine hemin (Fluka) and 25 mM Hepes to a pH of 5.8 and supplemented with 20% heat-inactivated FBS. The heterozygote LiSIR2^{+/−} derived from the wt clone was generated as previously described [30], and maintained in their respective selective medium. The levels of LiSIR2 synthesis in LiSIR2^{+/−}

and wt parasites were evaluated by Western blotting, using an anti-LiSIR2 monoclonal antibody as described previously [30].

Heterologous expression and purification of LiSIR2RP1

The LiSIR2RP1-encoding sequence (GenBank accession number AF487351) from *L. infantum* genomic DNA was amplified by PCR using two primers, a forward primer (5'-CAATTTGCATATGACAGCGTCTCCGAGAGCGCC A-3') and a reverse primer (5'-CCCAAGCGAATTCTCACGTCTCAT TCGGCGC-3'). The amplified product of approx. 1200 bp was cloned into the NdeI and EcoRI restriction sites of the prokaryotic expression vector pET28a (Novagen) and the fusion protein LiSIR2RP1 with an N-terminal tail of six histidines was produced in *Escherichia coli* BL21 (DE3). The transformants were grown in a Luria Broth medium containing 30 µg/ml kanamycin, and the recombinant protein expression was induced in mid-logarithmic phase with 1 mM IPTG (isopropyl β -D-thiogalactoside) for 3 h at 37°C. The bacterial pellet was re-suspended in a buffer containing 500 mM NaCl and 20 mM Tris/HCl (pH 7.6) and disrupted by sonication. The clarified supernatant was applied to a Ni-NTA (Ni²⁺-nitrilotriacetate) agarose column (Qiagen) and the recombinant LiSIR2RP1 protein was eluted with an imidazole gradient from 25 to 1000 mM. The eluted fractions were controlled for the presence of LiSIR2RP1 using SDS/PAGE. The fractions containing the recombinant protein were pooled, applied to PD-10 columns (Amersham Pharmacia Biotech) and eluted with PBS (pH 7.4).

Deacetylase assay

The NAD⁺-dependent deacetylase activity was evaluated by using a commercially available CycLex SIRT1/Sir2 deacetylase fluorometric assay kit (CycLex). The enzymatic reaction containing 0.25 µg of rLiSIR2RP1 (recombinant LiSIR2RP1) was carried out in accordance with the manufacturer's protocol and was monitored every 30 s for 1 h. The results are expressed as the rate of reaction for the first 20 min, due to the linear correlation between the fluorescence and this period of time.

Tubulin deacetylation assay

The tubulin deacetylation reactions were performed using either purified tubulin (Pure tubulin; Cytoskeleton) or *L. infantum* promastigote and axenic amastigote extracts, prepared in PEM buffer [80 mM Pipes (pH 6.8), 1 mM MgCl₂ and 1 mM EGTA] by sonication. In both cases, the tubulin was either left as heterodimers, or polymerized in PEM buffer in the presence of 20 µM taxol (Cytoskeleton) and 1 mM GTP (Cytoskeleton) for 45 min at 37°C. The reactions were carried out in deacetylase buffer [10 mM Tris/HCl (pH 8.0) and 10 mM NaCl], in the presence or absence of 1 mM NAD⁺ with constant agitation at room temperature (??°C) for 3 h. The reactions were stopped by adding 5 × Laemmli sample buffer and the proteins separated by SDS/PAGE (10% gels) and Western blotted.

ADP-ribosylation assays

The ADP-ribosylation assays were performed according to Garcia-Salcedo et al. [22]. Briefly, the assays were carried out with 1 µg of either of the following proteins: rLiSIR2RP1, rSIRT1 (recombinant human SIRT1; in the CycLex kit), or with the recombinant LicTXNPx (*L. infantum* cytosolic peroxiredoxin), in the presence of 5 µCi of [³²P]NAD and 5 µg of the following acceptor proteins: calf thymus histones (Sigma) or BSA (Sigma). Some assays were conducted with 0.5 µg of rLiSIR2RP1 or rLicTXNPx in the presence of 2.5 µCi of [³²P]NAD and 1 µg

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of pure tubulin or calf thymus histones. The reactions were performed at room temperature for 2 h in a buffer containing 150 mM NaCl, 10 mM DTT (dithiothreitol) and 50 mM Tris/HCl (pH 8.8), stopped with Laemmli loading buffer and dried in Whatman paper after electrophoresis, and then exposed to a film for 12 h at -80°C . For quantitative experiments, the reaction products were precipitated with 20 % TCA (trichloroacetic acid), washed and counted after the addition of 200 μl of scintillation fluid.

Antibodies

Antibodies to LiSIR2RP1 were obtained from BALB/c mouse immunization. The mouse was injected intraperitoneally with 50 μg of rLiSIR2RP1 in 200 μl of PBS three times at 7 day intervals. At 2 weeks after the final immunization, the serum was collected. The mouse monoclonal antibody, IIIG4, against the LmSIR2 (*L. major* SIR2), was obtained according to Vergnes et al. [29]. Mouse monoclonal anti- α -tubulin antibody (clone DM1A) and rabbit polyclonal anti- α -tubulin antibody were purchased from NeoMarkers. Mouse monoclonal anti-acetylated tubulin antibody (clone 6-11B-1) were obtained from Sigma. Rabbit anti-*T. brucei* RAB11 antibodies were a gift from Professor MC Field (???)

Cytoskeleton preparation

The cytoskeleton preparation was performed according to Schneider et al. [32]. Briefly, the *L. infantum* promastigotes and axenic amastigotes were collected and washed three times in ice-cold PBS. The parasites were resuspended at a concentration of 4×10^7 cells/ml in a buffer containing 10 mM Mops (pH 6.9), 0.1 mM EGTA, 1 mM MgSO_4 and 0.1 % Triton X-100 supplemented with protease inhibitor cocktail (Roche), and incubated on ice for 10 min followed by centrifugation at 3000 g for 5 min. The supernatant was collected and concentrated to one-tenth of the initial volume, and a 0.25 volume of $4 \times$ Laemmli sample buffer was added. The pellet was washed once with PBS and resuspended in 1/20 of the initial volume with a 1:1 mixture of PBS/Laemmli sample buffer. For immunofluorescence, the pellets were prepared as described below and after being washed with PBS, they were attached to poly-L-lysine-coated slides.

Immunofluorescence assays

L. infantum promastigotes and axenic amastigotes were fixed with 4 % paraformaldehyde in PBS for 1 h at room temperature. After several washes, the parasites were made permeable with 0.1 % Triton X-100 in PBS and incubated for 30 min in PBS-1 % BSA (PBS containing 1 % BSA). Parasites were then incubated with primary antibodies diluted in PBS-1 % BSA for 1 h at room temperature, washed several times with PBS, and then incubated for 1 h with the Alexa Fluor[®]-labelled secondary antibodies (Molecular Probes) diluted in PBS-1 % BSA at room temperature. The immunofluorescence assays using *Leishmania* cytoskeletons were performed as described above, with the exception of the fixation process, which was performed using 100 % methanol at 20°C for 15 min, and the permeabilization step, which was not performed. Washed parasites or cytoskeletons were mounted in Vectashield with DAPI (4',6-diamidino-2-phenylindole; Vector Laboratories). Z-series optical sections were collected through the Zeiss Axio Imager Z1 microscope (Carl Zeiss), using an AxioCam. Data stacks were deconvolved using either the Axiovision AxioVs40 V 4.2.0.0 (Carl Zeiss) or the Huygens Essential version 3.0.2p1 (Scientific Volume Imaging). Images were treated using Adobe Photoshop CS (Adobe Microsystems).

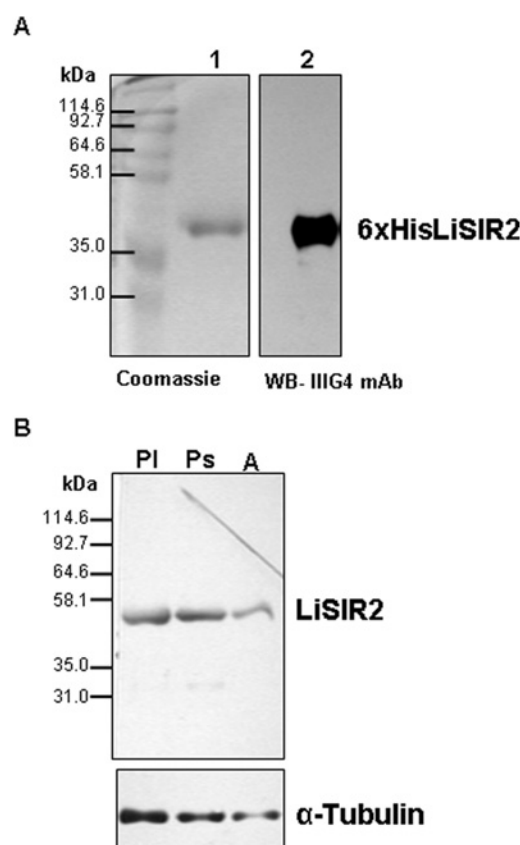


Figure 1 LiSIR2RP1 characterization

(A) The purity of the rLiSIR2RP1 was confirmed by SDS/PAGE Coomassie Blue staining (lane 1). A monoclonal antibody IIIG4 to recombinant LmSIR2RP1 showed strong positive signals when reacted in Western blot (WB) against rLiSIR2RP1 (lane 2). (B) Western blot analysis of LiSIR2RP1 expression in total parasite extracts shown using a mouse polyclonal antibody raised against the rLiSIR2RP1. Samples of protein extracts from each parasite developmental stage also reacted with the anti- α -tubulin monoclonal antibody, DM1A. PI, logarithmic promastigotes; Ps, stationary promastigotes; A, amastigotes. The molecular mass in kDa is indicated on the left-hand side.

RESULTS

LiSIR2RP1 is an NAD^+ -dependent deacetylase and ADP-ribosyltransferase

The LiSIR2RP1 gene-encoding sequence was cloned into the bacterial expression vector pET28a. The fusion protein with an N-terminal tail of six histidine residues was purified under non-denaturing conditions, with the objective of studying its enzymatic activity. After purification and dialysis, the protein showed a good level of purity as evaluated by SDS/PAGE Coomassie Blue staining (Figure 1A, lane 1). As expected, the recombinant protein was recognized by the monoclonal antibodies IIIG4 (anti-LmSIR2 protein), previously generated by our group (Figure 1A, lane 2) [29]. Moreover, the mouse polyclonal antibodies produced against the rLiSIR2RP1 recognized the parasite protein in total extracts from different parasite developmental stages [Figure 1B (PI, logarithmic phase promastigotes; Ps, stationary phase promastigotes; A, axenic amastigotes)]. It is well-established that the proteins belonging to the SIR2 family exhibit NAD^+ -dependent deacetylase activity due to the presence of a well-conserved enzymatic core domain of ~ 250 amino acids [7,8], and this seems also to be the case for LiSIR2RP1. Indeed, as shown in Figure 2(A), LiSIR2RP1 expressed an NAD^+ -dependent deacetylase

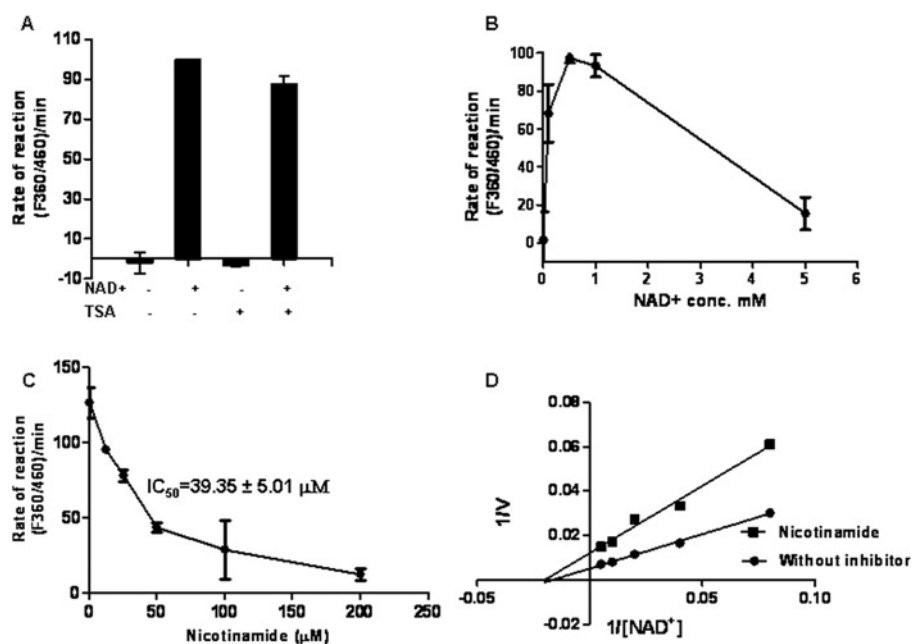


Figure 2 LiSIR2RP1 is a NAD⁺-dependent deacetylase

The NAD⁺-dependent deacetylase activity was measured using a fluorometric deacetylase kit in the presence of 0.25 μg of rLiSIR2RP1. LiSIR2RP1 is an NAD⁺-dependent deacetylase that is not inhibited by 1 μM of TSA (A). The rate of the deacetylase reaction was measured in the presence of several NAD⁺ concentrations (B). Nicotinamide, a deacetylase reaction product, inhibited the LiSIR2RP1 NAD⁺-dependent deacetylase activity in a dose-dependent manner (C) and according to the Lineweaver–Burk plot, 50 μM of nicotinamide promotes an NAD⁺ non-competitive inhibition (D). The results are expressed as the mean rate of reaction ± S.D. for the first 20 min.

activity that was not inhibited by 1 μM of the class I and II NAD⁺-independent deacetylase inhibitor, TSA (trichostatin A). A dose-dependent increase of the deacetylase activity catalysed by rLiSIR2RP1 was observed with increasing concentrations of NAD⁺ up to ≈0.5 mM. However, concentrations of NAD⁺ higher than 1 mM led to a reduction in the deacetylase activity (Figure 2B). This phenomenon is likely to be due to the release of nicotinamide, which in turn could inhibit the LiSIR2RP1 enzymatic activity (see below). This enzyme exhibits co-factor specificity for the oxidative form of NAD that was confirmed by the absence of deacetylase activity in the presence of the reduced form of NAD (results not shown). Nicotinamide is one of the several products originated in the deacetylation reaction catalysed by the SIR2 family members. As shown in Figure 2(C), the rLiSIR2RP1 was inhibited by nicotinamide in a dose-dependent manner, and the calculated nicotinamide concentration that inhibited 50% of the enzymatic activity (IC₅₀) was 39.35 ± 5.01 μM. Several enzymatic reactions in the presence or absence of 50 μM nicotinamide and different NAD⁺ concentrations were performed to determine whether NAD⁺ and nicotinamide were competing for the same binding site. The Lineweaver–Burk plot of the data demonstrates that nicotinamide is an NAD⁺ non-competitive inhibitor (Figure 2D).

Some members of the SIR2 family can mediate the transfer of the ribose 5'-phosphate from nicotinic acid mononucleotide to the amino acid residues of BSA, histones or SIR2 proteins themselves [7,21–24]. To assess whether LiSIR2RP1 is an ADP-ribosyltransferase, [³²P]NAD was used as a donor protein and BSA or calf thymus histones as acceptor proteins. A non-related *Leishmania* recombinant protein, rLicTXNPx1, which is produced and purified as the rLiSIR2RP1, was included as a control. The analysis of the reaction products by autoradiography revealed that LiSIR2RP1 was capable of inducing ADP-ribosylation of the histones and BSA (Figure 3A). However, the label intensity was

stronger when histones, rather than BSA, were used as acceptor proteins. Additionally, the parasite protein was subject to auto ADP-ribosylation when incubated with the histones. No signal was detected when the proteins were incubated with [³²P]NAD without an acceptor protein. A quantitative ADP-ribosyltransferase reaction catalysed by rLiSIR2RP1 using histones as the acceptor protein was carried out to characterize the factors affecting the extent of the enzymatic reaction. Increasing the concentration of rLiSIR2RP1 (2-fold) led to a decrease in the extent of the enzymatic transfer of the label, whereas a 2-fold increase of histone concentration led to an enhancement of ADP-ribosyl transfer to the acceptor target protein (Figure 3B). This unexpected reduction of the ribosylation reaction when adding more enzyme, might be due to some level of protein aggregation. In fact, we have experienced the purification of recombinant LiSIR2RP1 enzyme and found that, after dialysis, increasing the concentration of LiSIR2RP1 may lead to the formation of some aggregates.

Given that LiSIR2RP1 is a cytosolic protein, we decided to verify whether it was capable of ADP-ribosylating tubulin. Thus purified tubulin or calf thymus histones as a control were incubated with LiSIR2RP1 or LicTXNPx in the presence of [³²P]NAD. The autoradiography showed a positive but weak labelling of tubulin and LiSIR2RP1 itself, when compared with the reaction where histones were used as substrates. Even though tubulin ADP-ribosylation is an already reported modification, this is the first evidence of such ADP-ribosylation transfer mediated by a protein belonging to the SIR2 family [33]. This modification seems to either inhibit the *in vitro* assembly of microtubules or induce microtubule depolymerization of assembled microtubules [33].

The LiSIR2RP1 is a microtubule-associated protein

We have previously reported that both LmSIR2RP1 and LiSIR2RP1 are cytosolic proteins [6,29]. A similar cytosolic

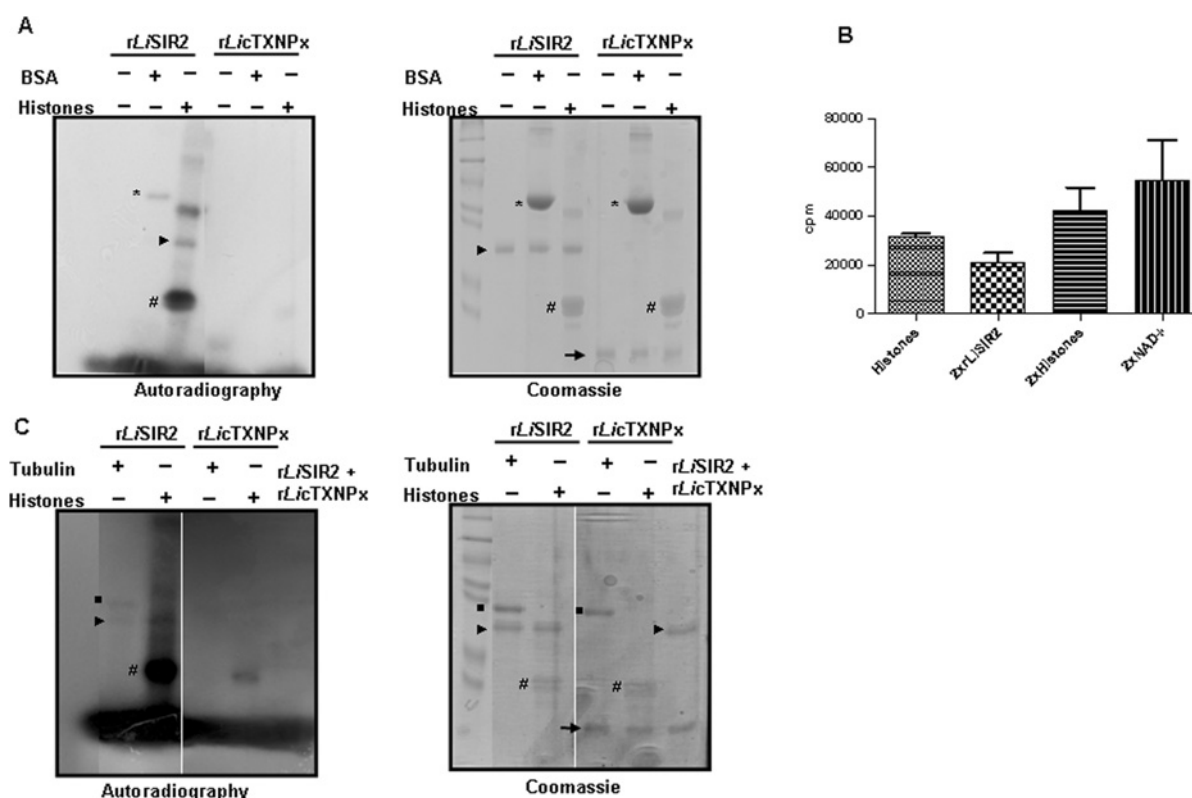


Figure 3 LiSIR2RP1 has ADP-ribosyltransferase activity

(A) The rLiSIR2RP1 (▶) ADP-ribosyltransferase activity was assessed using [³²P]NAD as the donor protein and BSA (*) or calf thymus histones (#) as acceptor proteins. A non-related *Leishmania* recombinant protein, rLiTXNPx1 (→), was included as a control. The reaction products were analysed by SDS/PAGE, and Coomassie Blue stained or autoradiographed. (B) Quantitative analysis of the effect of NAD⁺, acceptor protein or rLiSIR2RP1 amount on the ribosylation reaction. The reaction products were precipitated with TCA, collected, washed and then counted after the addition of scintillation liquid. (C) Assessment of tubulin (■) ADP-ribosylation mediated by rLiSIR2RP1. Tubulin and histones were included as acceptor proteins, [³²P]NAD as a donor protein and rLiTXNPx as the control. The reaction products were analysed by SDS/PAGE and were Coomassie Blue stained or autoradiographed.

localization was reported in the case of human SIRT2 (yeast SIR2 orthologue) and a human NAD⁺-independent HDAC, HDAC6, both proteins have been shown to express α -tubulin deacetylase activity [15,34]. Based on these observations and on the fact that tubulin is one of the most notable cytoplasmic proteins subjected to modification by acetylation [35], we first investigated the cellular distribution of LiSIR2RP1 and α -tubulin. The immunostaining of both promastigote and amastigote forms revealed partial co-localization of α -tubulin with LiSIR2RP1 (Figure 4A). In addition to the positive signals observed in the parasite cytoplasm, punctate labelling could be seen on the periphery of both promastigote and amastigote stages. This observation suggests the possible association of LiSIR2RP1 with the cytoskeleton. In order to examine this possibility, attempts were made to isolate the parasite cytoskeleton according to the method described by Schneider et al. [32], that allows subpellicular microtubules and the flagellar axoneme microtubules to be separated from the membrane and cytosolic components by a non-ionic detergent extraction using Triton X-100, followed by centrifugation. The cytoskeleton preparations were then reacted with the anti-LiSIR2RP1 antibodies. The results shown in Figure 4(B) suggest that part of LiSIR2RP1 is associated to the cytoskeleton network of both promastigote and amastigote forms. Interestingly, punctate labelling could also be seen over the flagellar axoneme microtubules when the cytoskeleton preparation was reacted with anti-LiSIR2RP1 antibodies. In agreement with this, the Western blot analysis of the detergent-soluble and insoluble fractions of promastigote and amastigote forms revealed, as expected, the pres-

ence of α -tubulin in the insoluble fraction, and of LiSIR2RP1 distributed in both soluble and insoluble fractions of promastigotes and amastigotes (Figure 4C). Although in the case of the promastigotes the detergent-insoluble fraction contains a band of ~64.6 kDa recognized by the anti-LiSIR2RP1 antibodies, its nature is presently unknown. It might be due to non-complete reduction of LiSIR2RP1 present in a molecular complex with other parasite polypeptides. As a control, we have used an antibody to *T. brucei* RAB11 that cross-reacts with *Leishmania* homologous protein already used by others to control the purity of cytoskeleton preparations of *L. major* [37]. Indeed, the RAB11 is a protein found on the membrane of endosomes not associated with the cytoskeleton [36]. As shown in Figure 4(A), the RAB11 has a different cell distribution pattern when compared with tubulin and LiSIR2RP1. Interestingly, no positive signal could be seen in immunofluorescence preparations of parasite cytoskeletons reacted with anti-RAB11 antibodies (Figure 4B). Moreover, the Western blot membranes dehybridized and re-probed with anti-RAB11 antibodies showed that the RAB11 protein was present exclusively in the detergent-soluble fraction of parasite extracts (Figure 4C), therefore supporting the specificity of the LiSIR2RP1 and α -tubulin interaction.

LiSIR2RP1 accumulates in the posterior pole of promastigotes after taxol treatment

Leishmania is a protozoan parasite characterized by a digenetic life cycle exhibiting a particular range of cell shapes mostly

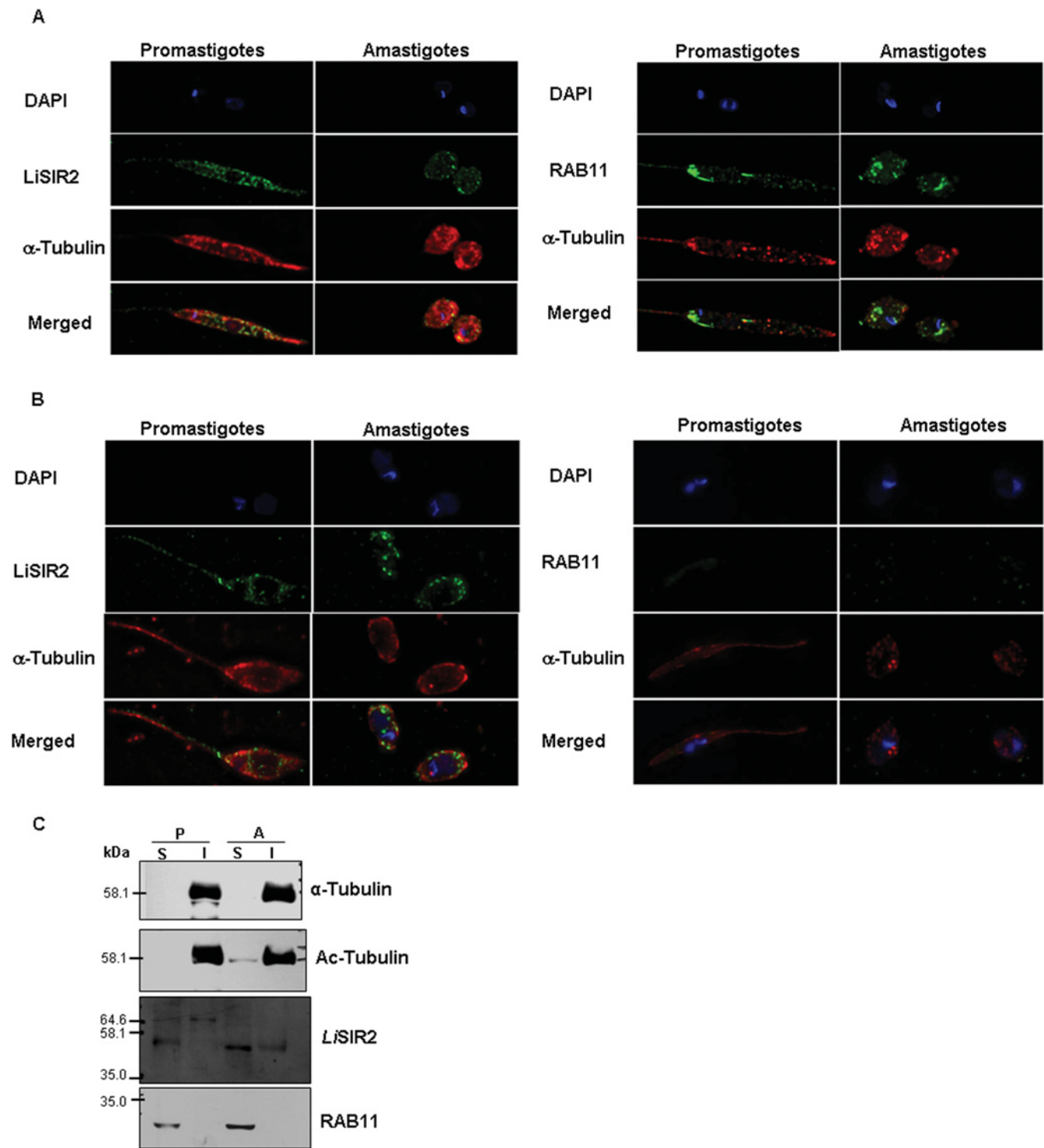


Figure 4 Cellular distributions of LiSIR2RP1 and α -tubulin

The *L. infantum* promastigotes and axenic amastigotes (**A**) and their respective cytoskeletons (**B**) were double stained by immunofluorescence with mouse anti-LiSIR2RP1 antibodies and rabbit anti- α -tubulin antibodies (left-hand panels) or with rabbit anti-TbRAB11 antibodies and mouse anti- α -tubulin antibodies followed by their respective Alexa Fluor® 488- (green) or Alexa Fluor® 568 (red)-conjugated IgG and counterstained with DAPI (blue). The parasites were visualized under a 1000 $\times$ magnification using a Zeiss Axio Imager Z1 microscope, and Z-series optical sections were collected using an AxioCam. Data stacks were deconvolved using either the Axiovision AxioVs40 V 4.2.0.0 (Carl Zeiss) or the Huygens Essential version 3.0.2p1. (**C**) Western blot analysis of Triton X-100-extracted *L. infantum* promastigote (P) and amastigote (A) fractions. S indicates the supernatant and I indicates the cytoskeleton pellet. The fractions were separated by SDS/PAGE (10 % gels), transferred to a nitrocellulose membrane and probed with either anti- α -tubulin, anti-acetylated tubulin, anti-LiSIR2RP1 or anti-TbRAB11 antibodies. The molecular mass in kDa is indicated on the left-hand side.

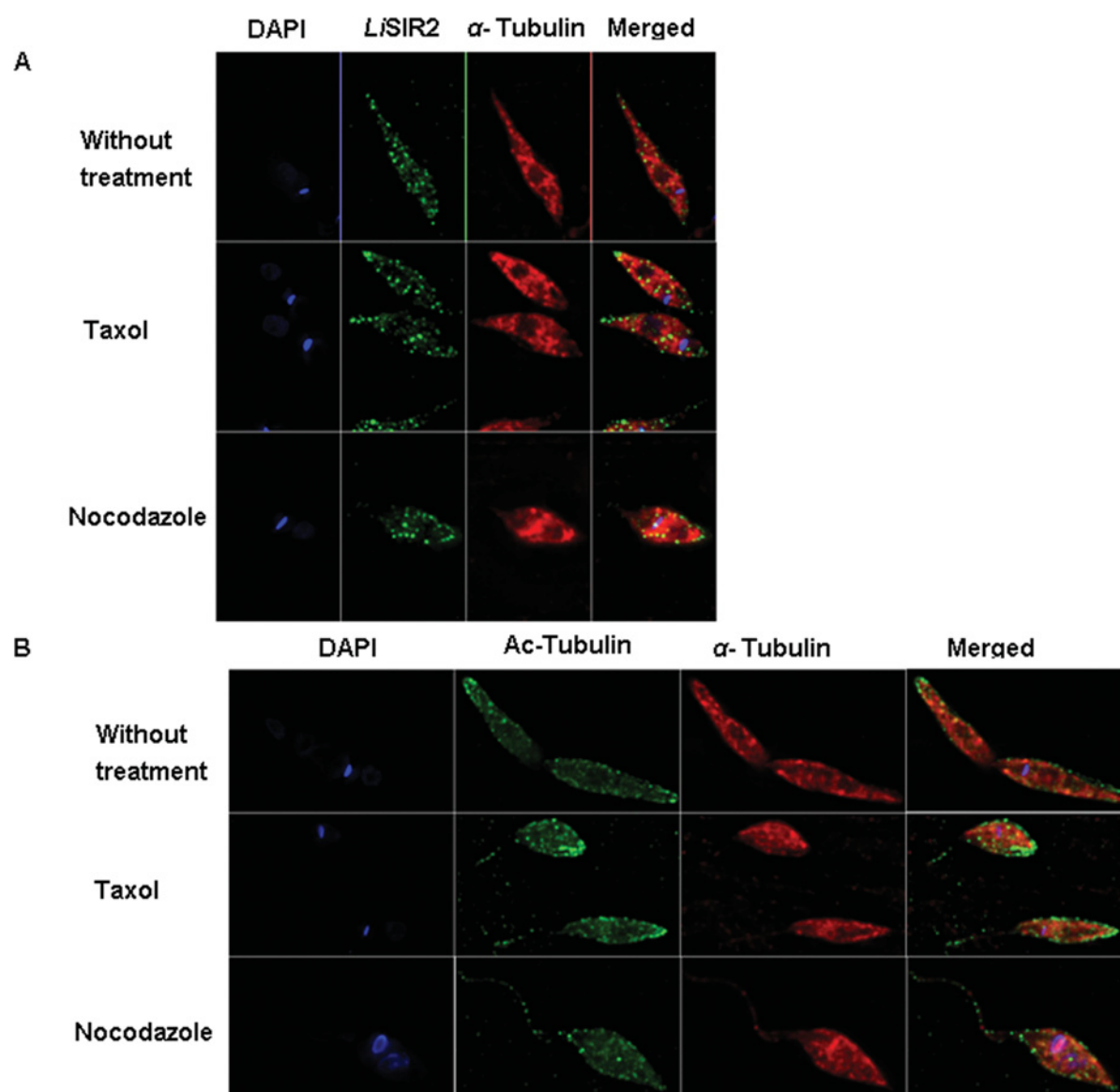


Figure 5 Cellular distribution of LiSIR2RP1, α -tubulin and acetylated α -tubulin after parasite treatment with taxol or nocodazole

The cellular distribution of the LiSIR2RP1, α -tubulin and acetylated α -tubulin were analysed after 16 h of *L. infantum* promastigote treatment with 1 μ M taxol or 5 μ g/ml nocodazole at 27°C. The parasites were fixed with paraformaldehyde, made permeable with Triton X-100 and double-labelled with mouse anti-LiSIR2RP1 antibodies and rabbit anti- α -tubulin antibodies (**A**) or with mouse anti-acetylated α -tubulin antibodies (6-11B-1) and rabbit anti- α -tubulin antibodies (**B**) followed by their respective Alexa Fluor® 488- (green) or Alexa Fluor® 568 (red)-conjugated IgG and counterstained with DAPI (blue). The parasites were visualized under a 1000 $\times$ magnification using a Zeiss Axio Imager Z1 microscope, and Z-series optical sections were collected using an AxioCam. Data stacks were deconvolved using either the Axiovision AxioVs40 V 4.2.0.0 (Carl Zeiss) or the Huygens Essential version 3.0.2p1.

defined by their internal cytoskeleton. The latter is composed mainly of microtubules that are polymers of repeating α/β tubulin heterodimers, and a variety of minor components known as microtubule-associated proteins. The microtubules play an important role in many cellular processes such as mitosis, cell shape, motility, intracellular vesicle transport and organelle position. Several compounds have been described as microtubule-interacting agents, based on their ability to induce or inhibit the tubulin assembly in microtubules. Even though a high degree of tubulin amino acid conservation is found throughout the evolution, there are some reports suggesting that colchicine and benzimidazoles, two classical mammalian tubulin inhibitors, had only a slight effect on the tubulin polymerization of trypanosomatids [38,39]. On the other hand, taxol, a microtubule-network-stabilizing agent,

affects the parasite cytoskeleton in a dose-dependent manner [40]. The *Leishmania* parasites were treated for 16 h with 1 μ M of the microtubule-stabilizing agent, taxol, or with 5 μ g/ml of the microtubule-network-disrupting agent, nocodazole, to analyse whether the SIR2RP1 cell distribution is affected by changes in the parasite cytoskeleton. Treatment of promastigotes with taxol led to a weak but distinguishable accumulation of SIR2RP1 in the posterior pole of the cell (Figure 5A). The treatment of parasites with nocodazole modified the tubulin distribution, being mainly localized near the nucleus in the parasite body. However, LiSIR2RP1 showed no particular modification in terms of cell distribution, compared with non-treated cells. Although the morphology of treated parasites differed from non-treated ones, their viability measured by PI (propidium iodide) incorporation

and flow cytometry analysis revealed no differences. Indeed, after 16 h of treatment with 1 μ M taxol or 5 μ g/ml nocodazole, the percentage of PI-negative parasites $\pm$ S.D. was 98.2 ± 0.1 and 97.6 ± 0.2 % respectively, compared with 97.4 ± 0.1 % for untreated ones. Interestingly, after taxol treatment, acetylated α -tubulin accumulated in the posterior pole of the cell (Figure 5B), coinciding with the high LiSIR2RP1-reactive zone seen in Figure 5(A). Moreover, punctate labelling could be seen on the whole periphery of the parasites as well as the flagellum. These observations are in agreement with the notion of LiSIR2RP1 and tubulin being interacting proteins.

LiSIR2RP1 deacetylates α -tubulin

In most eukaryotes, tubulin is subject to several post-translational modifications, including deetyrosination, acetylation, phosphorylation, palmitoylation, polyglutamylation and polyglycylation [41]. Tubulin acetylation occurs on Lys⁴⁰ of the α -tubulin subunit [42]. To test the ability of LiSIR2RP1 to deacetylate α -tubulin *in vitro*, several amounts of rLiSIR2RP1 were incubated with purified tubulin in the presence or absence of NAD⁺. The deacetylation of α -tubulin was analysed by Western blot with specific antibodies to either total α -tubulin or its acetylated form. As shown in Figure 6(A), incubation of a defined amount of tubulin (0.5 μ g) with increasing quantities of rLiSIR2RP1 (0.125–0.5 μ g) led to a dose-dependent deacetylation of α -tubulin. Interestingly, the addition of increasing concentrations of nicotinamide, a known physiological inhibitor of SIR2 deacetylases, led to significant inhibition of tubulin deacetylation by rLiSIR2RP1.

We further explored whether LiSIR2RP1 was able to deacetylate tubulin present within polymerized microtubules. Thus addition of taxol and GTP to tubulin heterodimers led to microtubule polymerization. Under these conditions, LiSIR2RP1 still had the capacity to efficiently deacetylate tubulin. Moreover, the addition of increasing concentrations of nicotinamide in the assay samples led to the inhibition of LiSIR2RP1-mediated tubulin deacetylation (Figure 6B).

We then examined whether this activity also occurred when using taxol- and GTP-treated parasite extracts as a source of polymerized tubulin. As shown in Figures 6(C) and 6(D), when total extracts from promastigotes and amastigotes treated with taxol were used as a source of polymerized microtubules, LiSIR2RP1 was also able to deacetylate tubulin. Taken together, these observations demonstrate that tubulin is a substrate of LiSIR2RP1 deacetylase.

We further examined the LiSIR2RP1 NAD⁺-dependent deacetylase substrate specificity by using acetylated BSA or cytochrome *c* and Western blotting. In contrast with tubulin, the level of BSA and cytochrome *c* acetylation presented no changes after incubation with LiSIR2RP1 in the presence or absence of NAD⁺. This allowed us to conclude that, at least from the approach we have used, LiSIR2RP1 does not deacetylate either BSA or cytochrome *c*, suggesting some substrate specificity of LiSIR2RP1 as a deacetylase (results not shown).

In order to evaluate whether LiSIR2RP1 was deacetylating tubulin *in vivo*, we have analysed the ratio of acetylated tubulin over total α -tubulin in the wt and single LiSIR2RP1-knockout promastigotes and axenic amastigotes. Only a slight difference in the level of tubulin acetylation between the wt and single-knockout axenic amastigotes was detected (Figure 6E). However, the disruption of a single allele from the LiSIR2RP1 gene may not be enough to affect the tubulin acetylation level, even though it is enough to reduce the ability of amastigotes to multiply, be it *in vitro* as free forms or inside macrophages, or *in vivo* in Balb/c mice [30].

DISCUSSION

In the present study, we describe and characterize, for the first time, the enzymatic activities of LiSIR2RP1. Assuming that this protein is involved in the virulence and survival of the parasite, it could constitute a new and potential drug target against leishmaniasis that needs to be exploited. We have demonstrated that, although LiSIR2RP1 differs from its *T. brucei* orthologue, TbSIR2RP1 [22] in terms of subcellular localization (cytosolic compared with nuclear), it expresses similar enzymatic activities, being able to act as an NAD⁺-dependent deacetylase and to transfer [³²P]NAD⁺ to histones and, to a lesser extent, to BSA or tubulin. Initial reports on the yeast SIR2 have shown that the protein has a weak mono-ADP-ribosyltransferase activity and a more robust NAD⁺-dependent deacetylase activity [21]. This finding led to the general idea that the ADP-ribosyltransferase activity might be the result of a non-enzymatic reaction. However, our results and the investigations using TbSIR2RP1 [22], the human SIRT6 [23] or the *P. falciparum* Sir2 [24], provided evidence supporting the notion that these SIR2 family members could mediate the enzymatic transfer of the ADP-ribosyl group. Studies from Kowieski et al. [43] have recently shed light on the ADP-ribosylation mechanism catalysed by the TbSIR2RP1. The authors demonstrated that ADP-ribosylation of histone by TbSIR2RP1 required an acetylated substrate. Additionally, two distinct ADP-ribosylation pathways that involve an acetylated substrate, NAD⁺ and TbSIR2RP1, were described. One is a non-catalytic reaction between the deacetylation product OAADPr [or its hydrolysis product, ADPr (ADP-ribose)] and histones, and the other a more efficient mechanism involving interception of an ADP-ribose-acetylpeptide-enzyme intermediate by a side-chain nucleophile from bound histone [43]. This might also explain the low level of tubulin ADP-ribosylation catalysed by the *Leishmania* SIR2 protein. If the enzyme preferentially deacetylates tubulin and the ADP-ribosylation requires an acetylated protein, the occurrence of the latter will be dependent on the first one [43].

The LiSIR2RP1 possesses the expected NAD⁺-dependent deacetylase activity that characterizes the SIR2 family members, and one of their reaction products, nicotinamide, is an NAD⁺ non-competitive inhibitor. Several biological activities have been attributed to the proteins belonging to the SIR2 family, that, even though sharing a conserved enzymatic core domain, are flanked at their N- and C-terminal parts by divergent sequences between family members, which may promote the binding to specific substrates [44]. Two main sirtuin substrates that have been identified are large fibrous macromolecular complexes, chromatin and tubulin [15]. The partial co-localization of LiSIR2RP1 with α -tubulin and its association with the parasite cytoskeleton, suggested that tubulin could be a substrate of this enzyme as it is for the human enzymes SIRT2 and HDAC6 [15,34]. Control tests using antibodies to a parasite cytosolic protein, RAB11, which confirmed the absence of a RAB11–tubulin relationship, strongly support the notion of a LiSIR2RP1–tubulin-specific interaction. In fact, LiSIR2RP1 was either able to deacetylate tubulin in dimers or when present in taxol-stabilized microtubules, or in total promastigote or amastigote extracts. Although the α -tubulin acetylation has already been reported, its importance in cell biological processes is not yet completely understood. The presence of acetylated tubulin in stable microtubules and the absence of highly acetylated microtubules in the fibroblast leading edge (a dynamic structure involved in cell motility), suggested that this post-translational modification could play a role in microtubule stability [45]. However, Palazzo et al. [46] demonstrated that enhanced tubulin acetylation does not increase the

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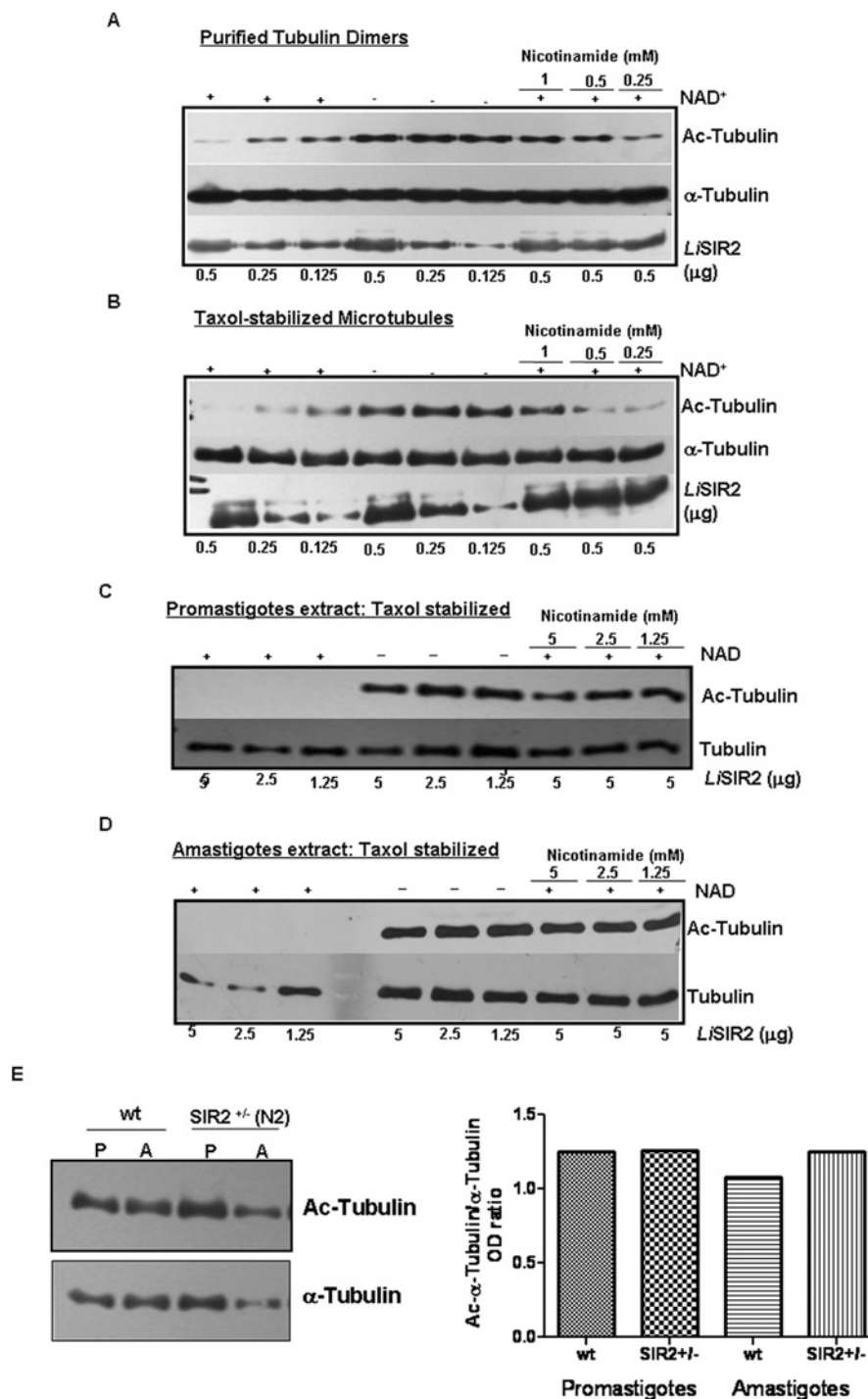


Figure 6 α -Tubulin is deacetylated by LiSIR2RP1

Purified tubulin dimers (A), taxol-stabilized microtubules (B), taxol-stabilized microtubules from whole promastigote lysate (C) and taxol-stabilized microtubules from whole amastigote lysate (D) were incubated with several amounts of purified rLiSIR2RP1 in the presence or absence of 1 mM NAD⁺ and several nicotinamide concentrations for 3 h at room temperature. The reaction products were analysed by Western blotting with specific antibodies to acetylated α -tubulin (Ac Tubulin), α -tubulin and LiSIR2RP1. (E) Analysis by Western blot of the acetylated α -tubulin (Ac-Tubulin) and total α -tubulin levels in the wt and LiSIR2^{-/-} mutant (clone N2) promastigote (P) and axenic amastigote (A) total extracts.

level of stable microtubules. Moreover, ciliated or flagellated protists can use a non-acetylatable α -tubulin with no observable ill effects on the microtubules or the organisms [47]. By contrast, the level of tubulin acetylation seems to affect a number of cellular events that includes the infection of CD4⁺ T-cells by HIV1 [48], the binding and transport of a kinesin 1 [49], the correct organ-

ization of the immune synapse [50], and the dynamics of cellular adhesions [51]. Interestingly, the promastigote treatment with the microtubule-stabilizing agent, taxol, increased the number of parasites showing an accumulation of LiSIR2RP1 in the posterior pole of the cell that is enriched in acetylated α -tubulin. It is likely that LiSIR2RP1 could bind to α -tubulin and just deacetylate it in a

specific step during the life cycle of the parasite. Additionally, the promastigotes infect macrophages through a receptor-mediated mechanism initiated at the posterior pole of the parasite [52]. One can hypothesize that the accumulation of LiSIR2RP1 in the posterior pole could be important to the contact site with the APC during the infection. Indeed, translocation and accumulation of HDAC6, a human cytosolic deacetylase, in the immune synapse at the contact site of T-cells with APC has previously been reported [50]. Furthermore, it is noteworthy that the influence of the deacetylase activity in the cell motility versus adhesion reported by Tran et al. [51] could also be relevant to the parasites when they are infecting the host cell.

The *Leishmania* parasites are subject to several modifications in their morphology during their complex life cycle. Although some environmental factors, such as pH, temperature and nutrients, can trigger morphological changes in the parasite, relatively little is known about the molecular processes that mediate the cellular remodelling. Further functional studies of LiSIR2RP1 could help to discover the role of the tubulin acetylation/deacetylation and/or ADP-ribosylation in the remodelling of the parasite's morphology and the infection of host cells.

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2.5 Differential effects of polyamine derivative compounds against *Leishmania infantum* promastigotes and axenic amastigotes

The natural polyamines are ubiquitous polycationic compounds that play important biological functions in cell growth and differentiation. In the case of protozoan species that are causative agents of important human diseases such as Leishmaniasis, an exogenous supply of polyamines supports parasite proliferation. In the present study, we have investigated the effect of three polyamine derivatives, (namely bisnaphthalimidopropyl putrescine (BNIPPut), spermidine (BNIPSpd) and spermine (BNIPspm)), on the proliferative stages of *Leishmania infantum*, the causative agent of visceral leishmaniasis in the Mediterranean basin. A significant reduction of promastigotes and axenic amastigotes growth was observed in the presence of increasing concentrations of the drugs, although the mechanisms leading to the parasite growth arrest seems to be different. Indeed, by using a number of biochemical approaches to analyse the alterations that occurred during early stages of parasite-drug interaction (i.e. membrane phosphatidylserine exposure measured by annexin V binding, DNA fragmentation, deoxynucleotidyltransferase-mediated dUTP end labelin (TUNEL), mitochondrial transmembrane potential loss), we showed that the drugs had the capacity to induce the death of promastigotes by a mechanism that shares many features with metazoan apoptosis. Surprisingly, the amastigotes did not behave in a similar way to promastigotes. The drug inhibitory effect on amastigotes growth and the absence of propidium iodide labelling may suggest that the compounds are acting as cytostatic substances. Although, the mechanisms of action of these compounds have yet to be elucidated, the above data show for the first time that polyamine derivatives may act differentially on the *Leishmania* parasite stages. Further chemical modifications are needed to make the polyamine derivatives as well as other analogues able to target the amastigote stage of the parasite.

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Differential effects of polyamine derivative compounds against *Leishmania infantum* promastigotes and axenic amastigotes

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Abstract

The natural polyamines are ubiquitous polycationic compounds that play important biological functions in cell growth and differentiation. In the case of protozoan species that are causative agents of important human diseases such as Leishmaniasis, an exogenous supply of polyamines supports parasite proliferation. In the present study, we have investigated the effect of three polyamine derivatives, (namely bis-naphthalimidopropyl putrescine (BNIPPut), spermidine (BNIPSpd) and spermine (BNIPSpm)), on the proliferative stages of *Leishmania infantum*, the causative agent of visceral leishmaniasis in the Mediterranean basin. A significant reduction of promastigotes and axenic amastigotes growth was observed in the presence of increasing concentrations of the drugs, although the mechanisms leading to the parasite growth arrest seems to be different. Indeed, by using a number of biochemical approaches to analyse the alterations that occurred during early stages of parasite-drug interaction (i.e. membrane phosphatidylserine exposure measured by annexin V binding, DNA fragmentation, deoxynucleotidyltransferase-mediated dUTP end labelin (TUNEL), mitochondrial transmembrane potential loss), we showed that the drugs had the capacity to induce the death of promastigotes by a mechanism that shares many features with metazoan apoptosis. Surprisingly, the amastigotes did not behave in a similar way to promastigotes. The drug inhibitory effect on amastigotes growth and the absence of propidium iodide labelling may suggest that the compounds are acting as cytostatic substances. Although, the mechanisms of action of these compounds have yet to be elucidated, the above data show for the first time that polyamine derivatives may act differentially on the *Leishmania* parasite stages. Further chemical modifications are needed to make the polyamine derivatives as well as other analogues able to target the amastigote stage of the parasite.

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Keywords: Polyamines derivative compounds; Apoptosis-like death; *Leishmania*

1. Introduction

In humans, parasites from the *Leishmania donovani* complex cause visceral leishmaniasis or Kala-azar (Zuckerman and Lainson, 1977). In Africa, *L. donovani* also infects adults but frequently presents a high resistance to antimonial treatment. Mediterranean Kala-azar, caused by *Leishmania*

donovani infantum, especially affects children and South American visceral leishmaniasis caused by *Leishmania donovani chagasi* is a disease of both adults and children. Geographical and clinical data have been used in the past to classify the organisms causing this visceral disease.

Chemotherapy is limited to the use of pentavalent antimonials such as sodium stibogluconate (Pentostan), N-methylglucamine (Glucantime), amphotericin B or pentamidine (Murry, 2001). However, due to the prolonged duration of therapy, causing adverse reactions and resistance to these compounds, the discovery of new antileishmanial compounds is required. The concept of drug resistance in

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Leishmaniasis is not straightforward; sensitivity to drugs has to be evaluated carefully and considered in relation to the differences in intrinsic drug sensitivity between species and zoonotic area. Treatment efficacy is also compromised in case of immunosuppression, in particular due to HIV co-infection. This can correspond to the exacerbation of the disease or emergence from latent infection due to the depletion of immune capacity and consequently unsuccessful standard chemotherapy (Berhe et al., 1999; Croft and Coombs, 2003; Watkins, 2003). Therefore, a search for new chemotherapeutic agents acting on parasite development is still warranted.

The natural polyamines, spermidine and spermine, and their precursor diamine putrescine, are present in most eukaryotic cells and have an important role in cell proliferation and differentiation (Muller et al., 2001). In trypanosomatid protozoa, polyamines have an additional role participating in the endogenous redox equilibrium through the compound (N1, N8-bis (glutathionyl) spermidine), named trypanothione T(S)2, which is maintained in its reduced dithiol form, T(SH)2, by trypanothione reductase (TR) (Fairlamb and Cerami, 1992). T(S)2 is the major redox reactive metabolite in trypanosomatids, this molecule and the enzymes involved in its metabolism are good drug targets (Fairlamb and Cerami, 1992; Barrett et al., 1999). Spermidine could be an essential polyamine needs for the maintenance of normal proliferation of trypanosomatid protozoa. This role is supported by the sensitivity of *L. donovani* cell lines deficient in the spermidine synthase gene (Roberts et al., 2001). Inhibition of proliferation occurs when the endogenous spermidine level was reduced to one third or less of its normal value, independently of the intracellular concentrations of putrescine (Gonzalez et al., 2001).

The intracellular concentrations of polyamines in mammalian cells are regulated by feedback mechanisms and involve multiple routes of synthesis and interconversion (Muller et al., 2001). Furthermore, targeting parasitic protozoan polyamines and their associated enzymes, like TR, ornithine decarboxylase, have become attractive targets for antiparasite therapy (Bonnet, 1997; Tovar et al., 1998; Muller et al., 2001). Therefore, it is reasonable to assume that selective interference with the parasitic polyamine metabolism will lead to an alteration of natural defence mechanisms of the parasite.

In fact, interference with the usual functions of polyamine has been one strategy in the search for effective antitumour drugs. The use of terminally alkylated polyamine analogues, able to mimic the natural polyamines in their self-regulatory role, but unable to act as substitutes for polyamines in their cell growth regulatory functions, has been shown to induce the growth inhibitory effects on several cancer cell lines (Ha et al., 1997; Dai et al., 1999; Davidson et al., 2000; Pavlov et al., 2002). In some cases, evidence reported showed that the polyamine analogues induced cell death via apoptosis seen by the fact that the dying cells displayed cell shrinkage, nuclear condensation

and fragmentation, though failing to obtain clear evidence of the DNA ladder, typical of apoptosis (McCloskey et al., 1995; Pavlov et al., 2002).

In this paper, we present the results of a comparative study of the effects of polyamine derivative compounds, assigned as BNIPPut, BNIPSpd and BNIPSpm, on the proliferative stages of *Leishmania infantum* (promastigotes and amastigotes). We show that the three compounds are potent antiproliferative agents against both forms of the parasite. However, analyses of the drug-induced alterations leading to parasite growth arrest revealed a killing process sharing many features with metazoan apoptosis only in the case of promastigotes whereas the activity against the amastigotes is likely due to a cytostatic effect.

2. Materials and methods

2.1. Polyamine compounds

The polyamine derivative compounds assigned as bis-naphthalimidopropyl putrescine (BNIPPut), spermidine (BNIPSpd), and spermine (BNIPSpm) were synthesised as described previously (Lin and Pavlov, 2000). Stock solutions of BNIPPut, BNIPSpd and BNIPSpm were prepared in 20% dimethylsulfoxide (DMSO) and stored at 4 °C. Working solutions were freshly diluted with culture medium until the desired final concentrations for the different assays were reached. The final concentrations of DMSO (0.02%) used did not interfere with any of the biological activities tested in this work.

2.2. Parasites

Leishmania infantum (clone MHOM/MA671TMA-P263) promastigotes were grown at 27 °C in RPMI medium (Gibco) supplemented with 10% heat inactivated fetal bovine serum (FBS-Gibco), 2 mM L-glutamine (Gibco), 20 mM Hepes (Gibco), 100 U/ml penicillin (Gibco) and 100 µg/ml streptomycin (Gibco) (Cordeiro-da-Silva et al., 2003). The parasites (10⁶/ml) in the logarithmic phase (2 days of culture) were incubated with a serial range of concentrations of each drug for 5 days at 28 °C. The growth of parasites was determined by three methods: (i) the counting in Newbauer chamber and by flow cytometry; (ii) by optical density at 600 nm; (iii) by methylthiazolotetrazolium (MTT) assay. The percentage of growth inhibition was calculated as (1-growth rate of the experimental culture/growth rate of the control culture) × 100. The IC₅₀, that is the concentration of the drug required to inhibit the growth by 50%, was determined by linear regression analysis.

Leishmania infantum axenic amastigote forms were grown at 37 °C with 5% CO₂ in a cell free medium called MAA/20 (medium for axenically grown amastigotes) (Serenio and Lemesre, 1997). MAA/20 consisted of

modified medium 199 (GIBCO) with Hank's balanced salt solution supplemented with 0.5% trypto-casein (Oxoid), 15 mM D-glucose (SIGMA), 5 mM glutamine (GIBCO), 4 mM NaHCO₃ (Sigma), 0.023 mM bovine hemin (FLUKA), and 25 mM HEPES to a final pH of 6.5 and supplemented with 20% heat inactivated FBS (Gibco). The parasites (2×10^5 /ml) were incubated with a serial range of concentrations of each drug for 5 days at 37 °C with 5% CO₂. The growth of parasites was determined by the same methods described above.

To monitor parasite death, short incubation periods were used. Promastigotes (10^6 /ml) or amastigotes (10^6 /ml) were treated with 10 µM of each drug for times ranging from 6 to 24 h. Different approaches were developed to explore the mechanisms of drug induced cell growth inhibition (see below).

2.3. Flow cytometric analysis of external phosphatidylserine exposure

Promastigotes or axenic amastigotes (1×10^6 /ml) were incubated with 10 µM of polyamine derivative compounds, for 22 h. Parasites were stained with FITC-conjugated annexin V, for 15 min, at room temperature, in a Ca²⁺ enriched binding buffer (apoptosis detection kit, R&D Systems). Then, propidium iodide (PI) was added to exclude the necrotic cells with disrupted plasma membrane permeability, and the parasites were analysed by flow cytometry (FACSCalibur, BD Biosciences). Promastigotes exposed to UV for 15 min were used as a positive control. Axenic amastigotes were also treated with 4 µM of staurosporine during 22 h and used as a positive control.

2.4. In situ TUNEL assay

DNA fragmentation was analysed in situ using a fluorescent detection system (In Situ Cell Death Detection Kit, Fluorescein-Roche). Slides containing promastigotes treated for 6, 12, and 24 h with polyamine derivative compounds and untreated promastigotes or amastigotes were fixed for 1 h with 4% paraformaldehyde (Merck) in PBS, washed with PBS, and stored at –20 °C until used. The TUNEL assay was conducted following the manufacturer's instructions. The parasites were observed under a fluorescent microscope (Axioskop-Carl Zeiss, Germany) at 400× magnification and images captured with a digital camera (Spot 2-Diagnostic Instruments, USA) and the software Spot 3.1 (Diagnostic Instruments, USA).

2.5. Flow cytometric analysis of DNA content

The DNA content of 1×10^6 /ml promastigotes or amastigotes treated and untreated with 10 µM of either of the three drugs during 24 h, was determined using propidium iodide after cell permeabilisation with 2% of saponin (Sigma) in PBS. Cells were analysed using FLH-3

detector and the Cell Quest software from a FACSCalibur (BD Biosciences).

2.6. Immunofluorescence assay

After 24 h treatment with 10 µM of polyamine derivative compounds, *L. infantum* promastigotes were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. After several washes, the parasites were permeabilised with 0.1% (v/v) Triton X-100 in PBS. Parasites were then incubated with a rabbit immune serum to a *L. infantum* recombinant mitochondrial protein belonging to the peroxiredoxin family (anti-LimTXNPx antibodies) (Castro et al., 2002) diluted 1:1000 in PBS containing 1% bovine serum albumin (PBS-BSA). The secondary antibody used was Alexa Fluor 488 goat anti-rabbit IgG diluted 1:2000 in PBS-BSA (Molecular Probes). Washed parasites were mounted in Vectashield (Vector Laboratories) and analysed with a fluorescent microscope (Axioskop-Carl Zeiss, Germany) at 1000× magnification and images captured with a digital camera (Spot 2-Diagnostic Instruments, USA) and the software Spot 3.1 (Diagnostic Instruments, USA). A negative control with untreated parasites was used.

2.7. Measurement of mitochondrial membrane potential in live *Leishmania* promastigotes or amastigotes

Tetramethylrhodamine ethylester perchlorate (TMRE, Molecular Probes) is a cationic lipophilic dye that accumulates in the negatively charged mitochondrial matrix according to the Nernst equation potential (Ehrenberg et al., 1988). A stock of TMRE was prepared at 4 mg/ml in DMSO and stored at –20 °C. For the determination of mitochondrial membrane potential, *L. infantum* promastigotes or amastigotes were incubated with 10 µM of BNIPput, BNIPspd or BNIPspm for 6, 12 or 24 h, washed once in PBS and resuspended at 2×10^6 cells/ml in PBS containing 100 nM TMRE. Cells were incubated for 15 and 30 min at room temperature and analysed by flow cytometry on the FL2-H channel. The PI at 4 µg/ml was used before the last acquisition to exclude dead cells. Propidium iodide (PI) fluorescence was recorded on FL3-H channel. As a positive control, cells already labeled with TMRE for 30 min were treated during 15 min with 200 µM final concentration of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, Sigma) during 15 min which depolarises mitochondria by abolishing the proton gradient across the inner mitochondrial membrane (Scaduto and Grotyohann, 1999).

2.8. Statistical analysis

The data were analysed using the Student's *t*-test. Probability values of < 0.01 or 0.05 were considered significant with 99 or 95% of confidence, respectively.

3. Results

3.1. The polyamine derivative drugs control the growth of *Leishmania infantum* parasites

Treatment of *L. infantum* promastigotes and axenic amastigote forms with BNIPPut, BNIPSpd or BNIPSpm at concentrations ranging from 0.125 to 25 μM (Fig. 1), resulted in a dose dependent inhibition of parasite growth, determined by quantification in a hemocytometer chamber (Fig. 2). It can be seen that at $\geq 12 \mu\text{M}$ all the three drugs completely blocked both promastigote and amastigote multiplication. However, below the above-mentioned concentration (down to 8 μM), BNIPPut and BNIPSpd were able to arrest promastigote growth. In the case of promastigotes, the calculated $\text{IC}_{50} \pm \text{SD}$ values for BNIPPut, BNIPSpd and BNIPSpm were 1.30 ± 0.24 , 1.9 ± 0.33 , and $7.56 \pm 0.04 \mu\text{M}$, respectively. The BNIPPut seems to be the most effective compound compared to BNIPSpd and BNIPSpm ($P=0.0635$; $P<0.01$). The effect of the three drugs on parasite axenic amastigote forms allowed us to determine the following $\text{IC}_{50} \pm \text{SD}$ values: 1.68 ± 0.06 , 4.56 ± 0.23 , and $12.28 \pm 1.30 \mu\text{M}$, for BNIPPut, BNIPSpd and BNIPSpm, respectively (Fig. 2). Although the IC_{50} values appear higher than those determined in the case of promastigotes, the BNIPPut remained the most effective drug ($P<0.01$) when comparing BNIPPut IC_{50} value to those obtained with BNIPSpd and BNIPSpm, respectively. Three other independent methods, counting in flow cytometry, MTT assay and optical density determinations, were used with similar results (data not shown).

3.2. Polyamine derivative drugs induce apoptosis-like DNA fragmentation in *Leishmania infantum* promastigotes

In order to determine whether the observed effect was due to a leishmanicidal activity and to establish the type

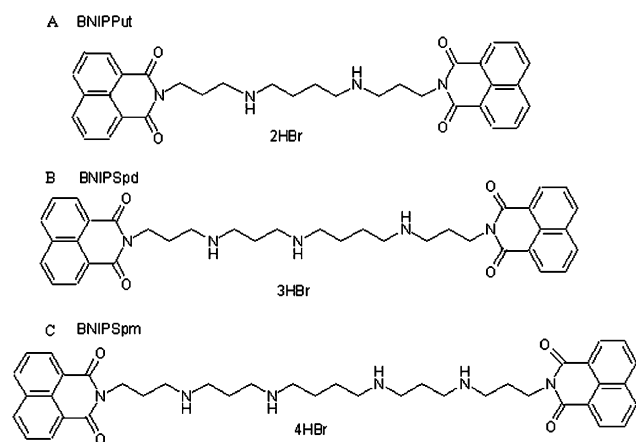


Fig. 1. Chemical structures of polyamine derivative compounds. Bis-naphthalimidopropyl putrescine (BNIPPut) (A), spermidine (BNIPSpd) (B) and spermine (BNIPSpm) (C).

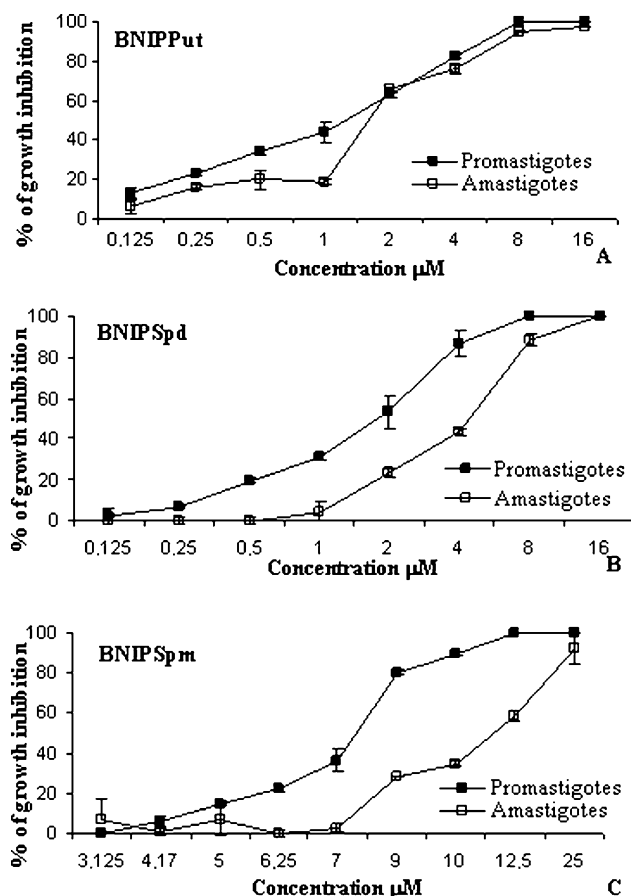


Fig. 2. Effect of different drugs on parasite growth in vitro. Representative inhibition growth curves of promastigotes and axenic amastigotes. Promastigote (closed square) and amastigote (open square) forms were incubated with a concentration range of 0.125–25 μM of bis-naphthalimidopropyl putrescine (BNIPPut) (A), spermidine (BNIPSpd) (B), and spermine (BNIPSpm) (C). Percentages of promastigote and amastigote growth inhibition were determined after 5 days by counting using a Newbauer chamber under light microscopy. Each point represents the mean of two replicates $\pm$ standard deviation. The data shown is representative of at least three independent experiments. The growth rate of the promastigote and amastigote parasite forms without drug treatment was 24 and 100 times related to the initial concentration, respectively.

of death that the drugs induced, promastigotes or amastigotes were incubated with 10 μM of each of the three compounds for 6, 12 and 24 h at 27 or 37 $^{\circ}\text{C}$, respectively. The changes occurring in the nuclear material was evaluated in situ by the TUNEL assay which detects the free ends of DNA after breakage. As shown in Fig. 3, in contrast to the parasites incubated without the drug where fewer labeled nuclei could be seen in Fig. 3A all promastigotes samples treated with either of the three drugs showed bright fluorescent nuclei indicating fragmentation of DNA (Fig. 3B–D). However, examination of a number of slides showed slightly different patterns of labelling. Indeed, the BNIPSpd induced fluorescent nuclei started to be visible only after 6 h of incubation. Moreover, only moderate

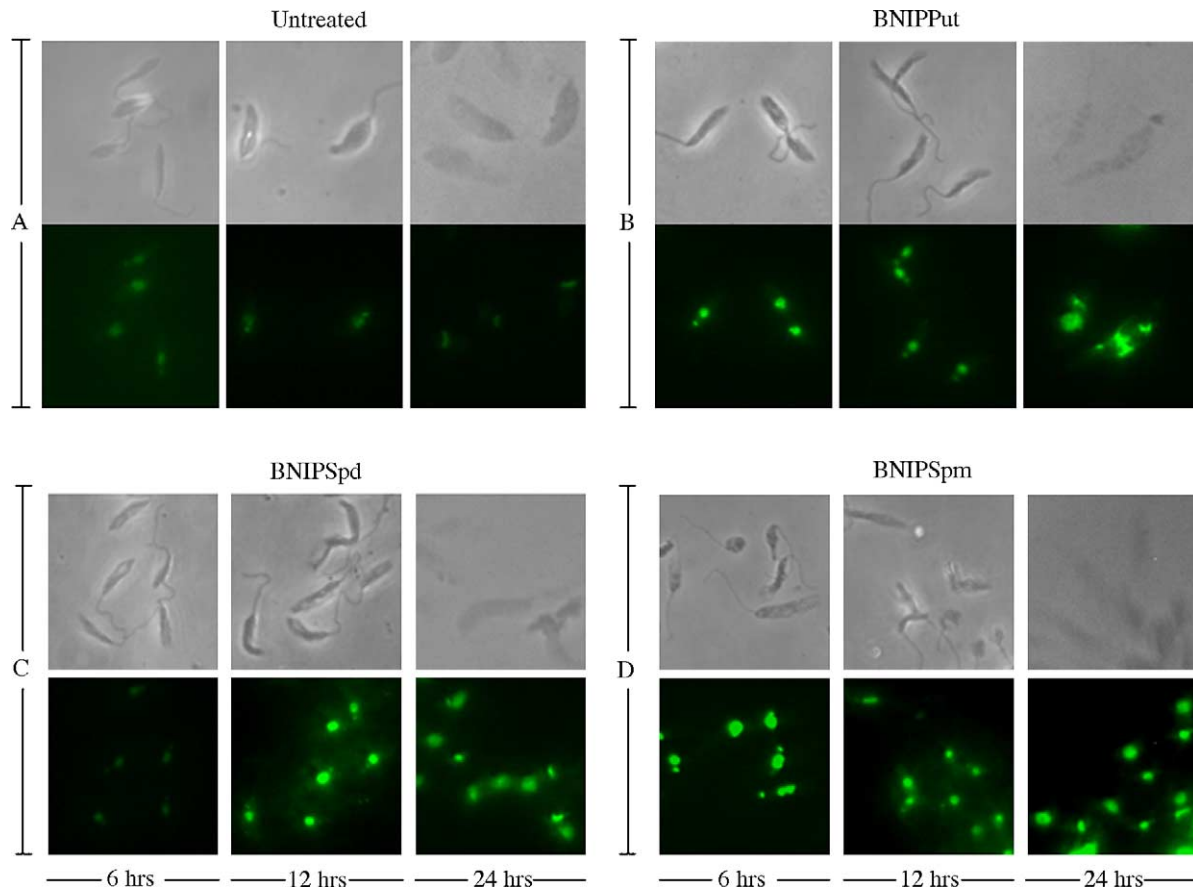


Fig. 3. In situ analysis of *Leishmania infantum* promastigotes DNA fragmentation. TUNEL assay (TUNEL, FITC) and phase contrast, were performed on logarithmic phase promastigotes. Untreated promastigotes (A) and parasites treated with 10 μ M of the polyamine derivative compounds bis-naphthalimidopropyl putrescine (BNIPPut) (B), spermidine (BNIPSpd) (C) and spermine (BNIPSpm) (D) for 6, 12, and 24 h, were submitted to the TUNEL assay as described under Section 2. The parasites were examined under a fluorescent microscope at 400 $\times$ magnification. The data are representative of three independent experiments.

fluorescent reactions could be seen in the case of BNIPPut (Fig. 3B) when compared to BNIPSpd (Fig. 3C) or BNIPSpm (Fig. 3C). As a positive control, parasite samples treated with DNase showed that almost all the nuclei and kinetoplasts were fluorescent (data not shown).

However, when studying the effect of the drugs on amastigotes, although some positive signals could be observed on some parasites after 6 and 12 h of incubation with either of the three drugs, no positive signals could be seen after 24 h of incubation (data not shown).

Since previous studies have shown that apoptosis-like death in trypanosomes and *Leishmania* parasites (Ouaisi, 2003) is associated with DNA fragmentation in nucleosome sized DNA fragments, we examined the occurrence of such phenomenon in genomic DNA samples from drug-treated parasites. We were unable to demonstrate any typical oligonucleosomal fragmentation in either promastigote or amastigote forms (data not shown).

We further examined by flow cytometry analysis after cell permeabilisation, the labeling with PI in order to quantify the percentage of pseudohypodiploid cells. The amount of bound dye is correlated with the DNA content in a given cell, and DNA fragmentation in apoptotic cells translates into a fluorescence intensity lower than that of G1 cells (sub-G1 peak) (Nicoletti et al., 1991). After 24 h of promastigote incubation with 10 μ M BNIPPut, BNIPSpd or BNIPSpm, the cells were found in the sub-G1 peak region, 34.82, 36.88, and 34.13%, respectively (Fig. 4B–D) compared with 7.64% in control cells (Fig. 4A), suggesting, therefore, that BNIPPut, BNIPSpd and BNIPSpm induced parasite DNA degradation. When the same approach was applied to the drug treated amastigotes, we could not observe similar profiles as those obtained in the case of promastigotes. Indeed, after 24 h of amastigotes incubation with 10 μ M BNIPPut, BNIPSpd (Fig. 4F) or BNIPSpm, the percentages of parasites found in the sub-G1 peak were 4.23, 5.55 and 3.81, respectively. These values are comparable with that

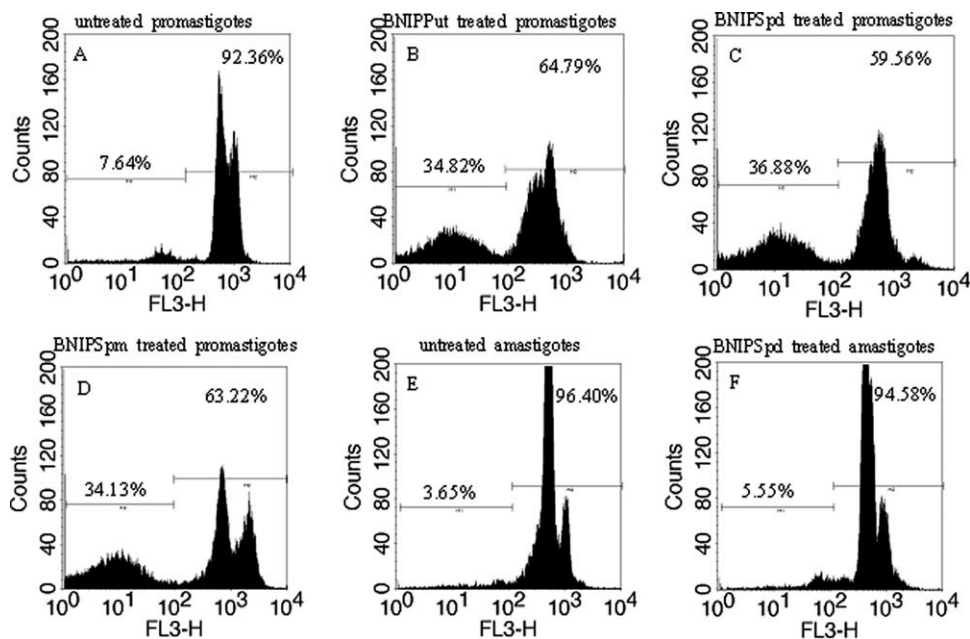


Fig. 4. Flow cytometry analysis of *Leishmania infantum* DNA content. Promastigotes or axenic amastigotes were incubated for 24 h in culture medium alone (A, E) or with 10 μ M of bis-naphthalimidopropyl putrescine (BNIPPut) (B), spermidine (BNIPSpd) (C, F) or spermine (BNIPSpm) (D). DNA content degradation was assessed by flow cytometry after cell permeabilisation and propidium iodide staining. Promastigotes incubated with: culture medium alone (A), BNIPPut (B), BNIPSpd (C) and BNIPSpm (D). Amastigotes incubated with culture medium alone (E) or culture medium plus BNIPSpd (F). The profile obtained with BNIPSpd is comparable to those obtained with BNIPPut and BNIPSpm (data not shown). The data shown are representative of three independent experiments.

observed in the case of amastigotes incubated in the culture medium alone (Fig. 4E).

3.3. Quantification of phosphatidylserine externalisation in *Leishmania infantum* promastigotes and amastigotes treated with polyamine derivative compounds

In mammalian cell apoptosis, a ubiquitous alteration is the translocation of phosphatidylserine from the inner side to the outer layer of the plasma membrane. Annexin V, a Ca^{2+} dependent phospholipid binding protein with affinity for phosphatidylserine, is routinely used in a fluorescein conjugated form to label externalisation of phosphatidylserine. Since annexin V-FITC can also label necrotic cells following the loss of membrane integrity, simultaneous addition of PI, which does not permeate cells with an intact plasma membrane, allows discrimination between apoptotic cells (annexin V positive and PI negative), necrotic cells (annexin V positive and PI positive) and live cells (annexin V negative and PI negative) (Vernes et al., 1995). *Leishmania infantum* promastigotes or amastigotes were incubated with 10 μ M of each compound for 22 h at 27 or 37 $^{\circ}\text{C}$, respectively and processed for phosphatidylserine externalisation measurement as indicated under Section 2. As shown in Fig. 5, increased expression of phosphatidylserine on the surface of drug-treated promastigotes was observed. Indeed, the percentages of Annexin positive parasites in the presence of the drug increased dramatically from 35.97 to 61.36% (Fig. 5C–E)

when compared to non-treated cells (1.72%, Fig. 5A). As a positive control UV exposed promastigotes showed 16.57% Annexin V positive cells (Fig. 5B).

In contrast to promastigotes, BNIPPut treated amastigotes showed no significant increase of Annexin-V positive cells (Fig. 5G) when compared to the non treated cells (Fig. 5F). Similar profiles of Annexin-V binding were obtained when axenic amastigotes were treated with either BNIPSpd or BNIPSpm (data not shown). This is likely due to the absence of apoptosis induction since amastigotes treated with 4 μ M staurosporine, a drug known to induce apoptosis-like death in *Leishmania* showed around 80% Annexin-V positive cells (Fig. 5H).

3.4. *Leishmania infantum* cell death is associated with the modification of membrane mitochondrial permeability and potential

Previous studies suggested that nuclear features of apoptosis in metazoan cells, like condensation of nuclei and fragmentation of DNA, are preceded by alterations in mitochondrial structure and transmembrane potential (Zamzami et al., 2001). Thus, we explored whether BNIPPut, BNIPSpd and BNIPSpm treatment caused an early response to the parasite mitochondrial membrane potential using TMRE fluorescent probe and FACS analysis. Thus, promastigotes or amastigotes were treated with BNIPPut, BNIPSpd and BNIPSpm for 6, 12, and 24 h and then incubated with the probe for 15 and 30 min.

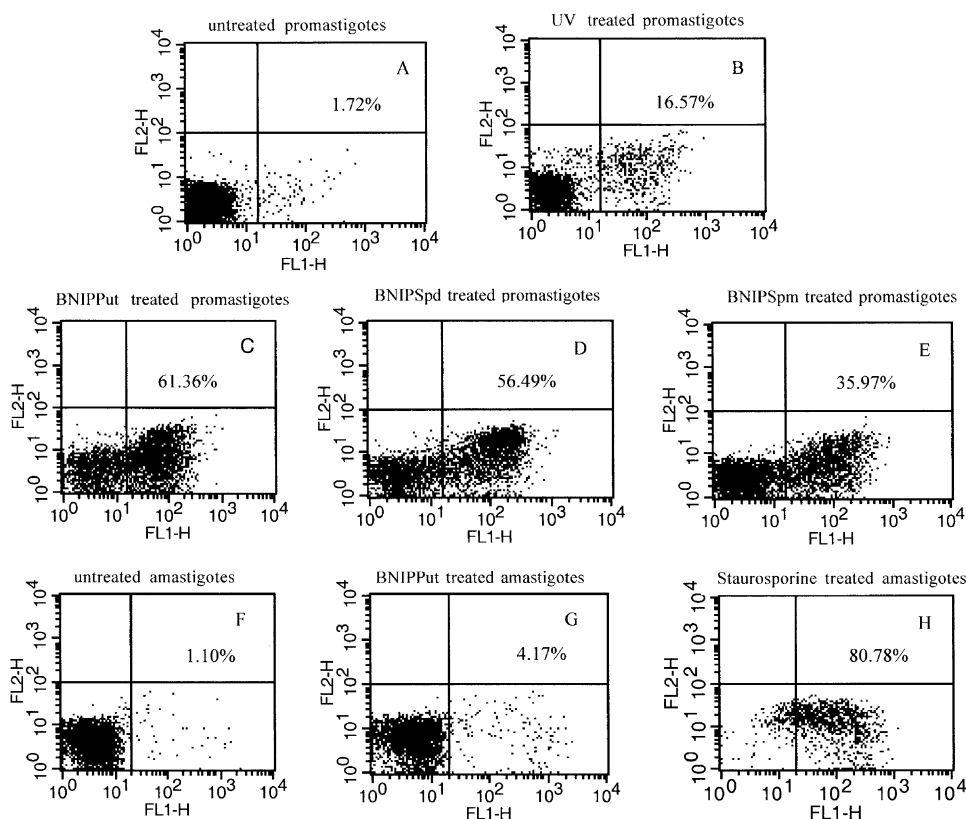


Fig. 5. Flow cytometry analysis of phosphatidylserine exposure to *Leishmania infantum* after polyamine derivative compound treatment. Promastigotes (A–E) and axenic amastigotes (F–H), were incubated in the absence (A, F) or presence of 10 μ M of polyamine derivative compounds: bis-naphthalimidopropyl putrescine (BNIPPut) (C, G), spermidine (BNIPSpd) (D) and spermine (BNIPSpm) (E) for 22 h. Phosphatidylserine exposure was monitored by flow cytometry. Dead cells were excluded by propidium iodide incorporation. Dot plots are representative of three independent assays. The promastigotes were UV exposed for 15 min (B) and amastigotes were treated with 4 μ M of staurosporine (H) during 22 h as a positive control. The profiles obtained with BNIPSpd and BNIPSpm treated amastigotes were similar to that shown for BNIPPut in G (data not shown).

Fluorescence of cells treated with TMRE and PI was measured by FACS analysis. Although we did not observe the modification of mitochondrial membrane potential after 6 h of drug treatment in both amastigotes and promastigotes, upon 12 h promastigote incubation with BNIPPut, BNIPSpd or BNIPSpm, a significant decrease in parasite mitochondrial membrane potential (31.18, 30.32 and 37.19%, respectively) occurred compared to the non treated parasites (98.14%, Fig. 6A). Moreover, after 24 h of BNIPPut, BNIPSpd and BNIPSpm incubation, similar profiles as those observed after 12 h treatment were recorded (data not shown).

In contrast to the promastigotes, we were unable to detect any modification of amastigotes mitochondrial membrane potential after 12 h, (Fig. 6B) or 24 h (data not shown) of treatment with 10 μ M of each of the three drugs.

In previous work, we found that the *L. infantum* mitochondrial protein, *LimTXNPx*, co-localise with the mitochondrial standard staining (Mito Tracker) (Castro et al., 2002). Therefore, we further examined the mitochondrial integrity by using the anti-*LimTXNPx* as a probe to determine the cellular distribution of *LimTXNPx* protein.

DAPI was also used to label the nucleus and kinetoplast DNA. After 24 h of incubation with 10 μ M of BNIPPut, BNIPSpd or BNIPSpm, the mitochondrial protein *LimTXNPx* showed cytoplasmatic localisation, whereas in the non treated cells the *LimTXNPx* remained inside the mitochondria (Fig. 7). These results reinforce the notion that drug treatment induced the alteration of mitochondrial membrane potential leading to apoptosis-like death of promastigotes.

4. Discussion

The polyamine family including putrescine, spermidine and spermine have important physiological roles (Gonzalez et al., 2001; Muller et al., 2001). On the basis of the vast array of biochemical functions and their role in cell growth and differentiation, several polyamine synthetic analogues have been used as tools for the study of the physiological roles of natural polyamines in terms of their antiproliferative properties (Thomas and Thomas, 2001). For several years one of us has been synthesising polyamine derivatives

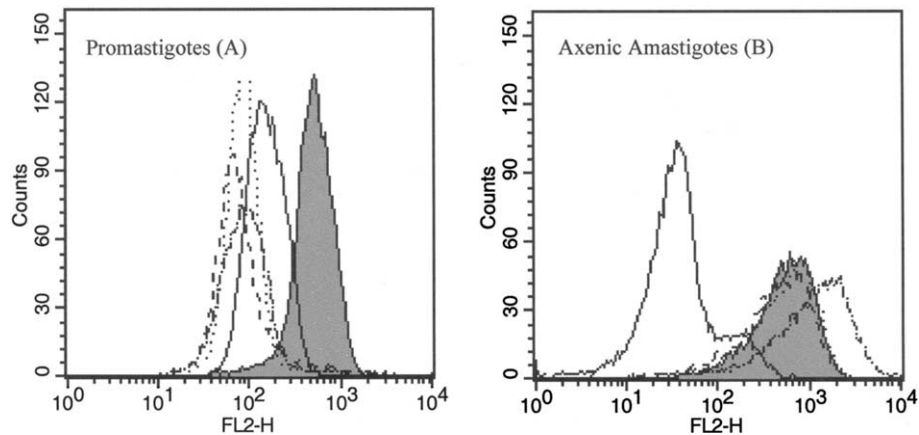


Fig. 6. Effect of bis-naphthalimidopropyl putrescine (BNIPPut), spermidine (BNIPSpd) and spermine (BNIPSpm) on the mitochondrial membrane potential as measured by the TMRE probe. Live *Leishmania* promastigotes (A) or axenic amastigotes (B) were treated with 10 μ M of BNIPPut (sketch line), BNIPSpd (dot line), and BNIPSpm (sketch and dot line) for 12 h. Untreated cells (full peak) showed positive control labelling, whereas the negative control consisted of parasites treated with the uncoupler CCCP (continuous line). The histogram shows fluorescence intensity of TMRE after 30 min of fluorescent probe time incubation. In the part B we have an overlay effect of BNIPPut (sketch line), and BNIPSpd (dot line) with the control (full peak).

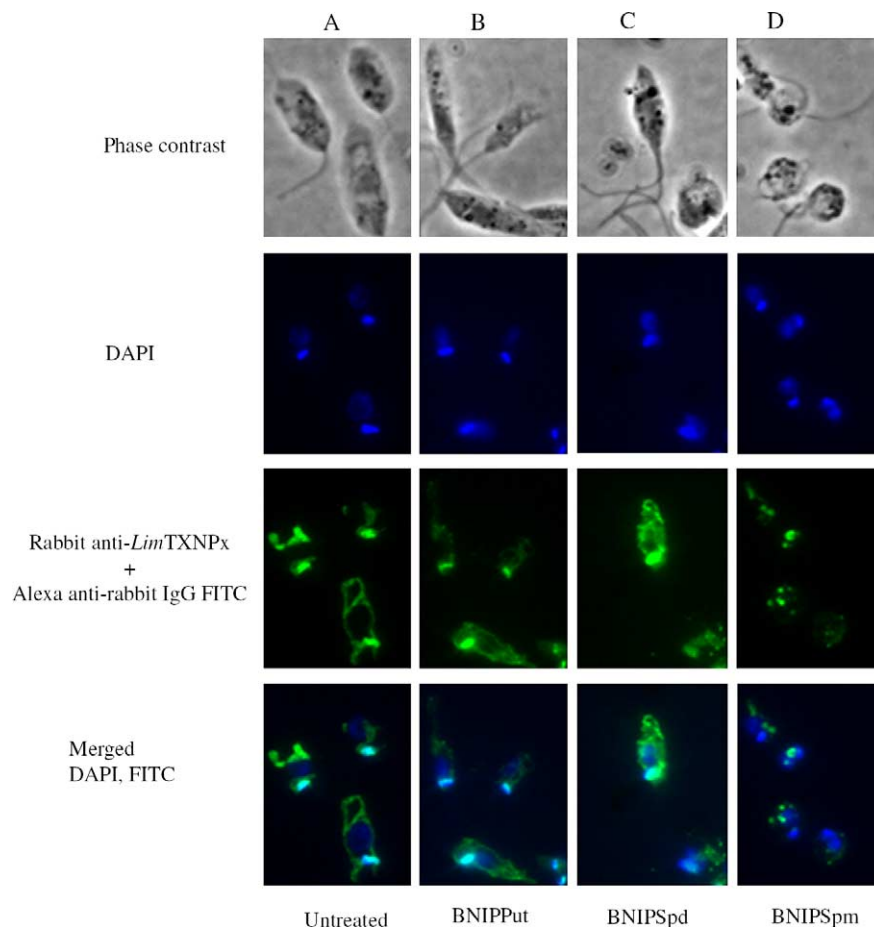


Fig. 7. Alteration of mitochondrial permeability by polyamine derivative drugs. The localisation of the mitochondrial protein *LimTXNPx* was analysed by immunofluorescence assay. *Leishmania infantum* promastigotes were treated with culture medium alone (A) or with 10 μ M of polyamine derivative compounds: bis-naphthalimidopropyl putrescine (BNIPPut) (B), spermidine (BNIPSpd) (C) and spermine (BNIPSpm) (D) for 24 h and reacted with the rabbit anti-*LimTXNPx* antibodies followed by Alexa Fluor 488 goat anti-rabbit IgG. The parasites were examined under a fluorescent microscope at 1000 $\times$ magnification. The data shown are representative of three independent experiments.

having the capacity to interfere with the growth of cancer cells. This kind of polyamine derivative compounds showed a high in vitro cytotoxicity against the human breast cancer MCF-7 cell line (Pavlov et al., 2000). Given that polyamines are known to be essential factors for the growth of the parasites in their host and that cancer cells and *Leishmania* parasites share at least one common feature, that is their mutual capacity for rapid cell division, we attempted to answer the following: first, whether the three polyamine derivatives, BNIPPut, BNIPSpd and BNIPSp, could exert an anti-parasite proliferative activity, and if so whether there is a phenotypic variance in cell growth arrest (i.e. leishmanio-static or leishmanolytic) and second, the type of cell death. Both antiproliferative and leishmanicidal effects, at least in the case of promastigotes, were observed for concentrations of these compounds in the micromolar range. The reductions in the proliferation of the promastigotes as well as the amastigotes, the vertebrate stage of the parasite, were dose dependent for all three drugs tested. However, comparing the structures of the three compounds, BNIPPut with the smallest polyamine chain, was found to be the most potent compound with IC₅₀ values of 1.30 and 1.68 μ M for promastigotes and amastigotes, respectively. The increase in the length of the polyamine chains leads to increased IC₅₀ values.

Using biochemical and morphological approaches, we showed that treatment with polyamine derivative compounds induces promastigotes death sharing phenotypic features with metazoan apoptosis (Ameisen, 2002; Zangger et al., 2002). Although we did not observe DNA ladders, a hallmark of metazoan apoptosis, similar observations have been reported by other investigators who failed to demonstrate a typical oligonucleosomal fragmentation of DNA from staurosporine treated *Leishmania major* parasites while showing a DNA degradation (hypoploidy) by flow cytometry (Arnoult et al., 2002). Furthermore, it is noteworthy that although the kinetoplastid parasites may share a death process with features reminiscent of mammalian nucleated cells apoptosis, the pathways (induction/execution) may differ at the molecular level (Ouaisi, 2003). Moreover, similar polyamine derivative compounds which induced apoptosis in cancer cells, failed to give DNA laddering (Gooch and Yee, 1999; Pavlov et al., 2002), suggesting that this may be due to the characteristics of these compounds.

Interestingly, the TUNEL staining clearly demonstrated that DNA fragmentation occurs in the promastigote nuclei during polyamine drugs induced cell death before membrane integrity is compromised, supporting the notion that the death process observed is not necrotic. Moreover, additional experiments performed showed that a number of features also found in apoptosis of multicellular organisms (i.e. cell shrinkage, phosphatidylserine exposure with preservation of plasma membrane integrity, and modification of mitochondria potential and permeability), also occurred in polyamine derivatives treated *L. infantum* promastigotes. Furthermore,

using an antibody probe directed against a *L. infantum* mitochondrial protein, LimTXNPx, we could demonstrate that mitochondrial membrane alterations occurred during parasite drug interaction. Surprisingly, although a growth inhibition could be clearly demonstrated in the case of drug treated axenic amastigotes, we were unable to observe any of the apoptosis-like features occurring in the drug treated promastigote forms. Since the drug treated amastigotes did not incorporate the PI, it is unlikely that the amastigotes were dying by necrosis. The simplest interpretation of this phenomenon could be that the drugs are exerting a cytostatic effect on the amastigote forms. Another possibility could be that the amastigotes develop some kind of mechanism allowing them to resist the apoptosis inducing effect of the drugs. Further studies are needed to clarify this point.

Parasites have distinct polyamine metabolisms compared to mammalian cells. Interfering with the parasite specific polyamine metabolism pathway will have more severe consequences for the parasite than for its host. Recent studies on parasitic protozoa have focused on the biochemical properties and susceptibility to inhibition by enzymes involved in the polyamine synthesis. Protozoa belonging to the trypanosomatid family such as *Leishmania*, synthesise a unique co-factor that is a conjugate of spermidine and glutathione. This co-factor, termed trypanothione, is required to maintain redox balance in the cell (Garforth et al., 1997). Different inhibitors of trypanothione reductase have antitrypanosomal activity (Bonnet et al., 2000). Thus, the parasite specific enzymes of trypanothione metabolism are considered attractive targets for drug development (Krauth-Siegel and Coombs, 1999). Further chemical modifications of the polyamine compounds is needed to target the amastigote, the vertebrate stage of the parasites.

In summary, our results indicate that polyamine derivative drugs, BNIPPut, BNIPSpd and BNIPSp induce an apoptosis-like death in *L. infantum* promastigotes and failed to exert similar effects on axenic amastigotes. Better understanding of the mechanisms of action of these polyamine derivative compounds together with the identification of the major pathways involved in *Leishmania* apoptosis-like death is essential to the development of new analogues which may target the amastigote, the vertebrate stage of the parasite.

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2.6 The synthesis and the *in vitro* cytotoxicity studies of bisnaphthalimidopropyl polyamine derivatives against colon cancer cells and parasite *Leishmania infantum*

Bisnaphthalimidopropyl derivatives (BNIPSpd, BNIPDaoct, BNIPDanon, BNIPDadec, BNIPDpta and BNIPDeta) were synthesised in yields ranging from 50% to 70% and their cytotoxicity against colon cancer cells (Caco-2) and the parasite *Leishmania infantum* determined using the MTT assay. Cytotoxicity within Caco-2 cells was manifested with IC₅₀ values between 0.3 and 22 µM. Compounds with the central longer alkyl chains exhibited the highest cytotoxicity. Against *L. infantum*, IC₅₀ values were encompassed within a narrower concentration range of 0.47–1.54 µM. In the parasites, the presence of nitrogen in the central chain and the length of the central alkyl chains did not especially enhance cytotoxicity. This may be due to the way these compounds are transported in the cells.

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The synthesis and the in vitro cytotoxicity studies of bisnaphthalimidopropyl polyamine derivatives against colon cancer cells and parasite *Leishmania infantum*

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Abstract—Bisnaphthalimidopropyl derivatives (BNIPSpd, BNIPDaoc, BNIPDanon, BNIPDadec, BNIPDpta and BNIPDeta) were synthesised in yields ranging from 50% to 70% and their cytotoxicity against colon cancer cells (Caco-2) and the parasite *Leishmania infantum* determined using the MTT assay. Cytotoxicity within Caco-2 cells was manifested with IC₅₀ values between 0.3 and 22 µM. Compounds with the central longer alkyl chains exhibited the highest cytotoxicity. Against *L. infantum*, IC₅₀ values were encompassed within a narrower concentration range of 0.47–1.54 µM. In the parasites, the presence of nitrogen in the central chain and the length of the central alkyl chains did not especially enhance cytotoxicity. This may be due to the way these compounds are transported in the cells.

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1. Introduction

Naphthalimido derivatives exhibit considerable potential as cytotoxic agents for cancer chemotherapy.^{1,2} We previously reported the synthesis and biological activities of a novel series of bisnaphthalimidopropyl polyamines.³ Subsequent work revealed the presence of the bisnaphthalimidopropyl functionality to be essential for optimum biological activity since the presence of an oxygen atom in the α -position of the naphthalimido ring tends to reduce activity.⁴ Majority research traditionally focused on the modification of the naphthalimido rings to enhance anticancer activities through

increased DNA binding and cleavage. For example, acenaphthalimide was introduced into the naphthalimide chromophore to increase the solubility of the bisnaphthalimide compounds.^{5,6} Furan heterocycles were added to the naphthalimide chromophore and those compounds exhibited strong DNA binding properties with toxicity to CEM leukaemia cells in the nanomolar concentration.⁷ Pyrazine heterocycles have also recently been fused to naphthalimides and those pyrazino-naphthalimides exhibited in vitro toxicity with IC₅₀ values ranging from 0.002 to 7.8 µM after 72 h treatment in cancer HT 29, HeLa and PC 3 cells.⁸

However, in our laboratory we have developed bisnaphthalimidopropyl fragments linked to natural polyamines such as putrescine, spermidine, and spermine. The spermidine and spermine derivatives exhibited enhanced aqueous solubility while maintaining good biological activity.⁹ In MCF 7 breast cancer cells, compounds were observed within the cell nuclei after 6 and 12 h drug exposure, with transport being potentially energy dependent. Within MCF7 cells, the bisnaphthalimidopropyl compounds inflicted significant quantitative DNA damage.¹⁰ Brana et al. reported similar qualitative observations of DNA damage in response to their bisnaphthalimidoethyl

Abbreviations: BNIPSpd, bisnaphthalimidopropylspermidine; BNIPDaoc, bisnaphthalimidopropyl-diamino-octane; BNIPDanon, bisnaphthalimidopropyl-diamino-nonane; BNIPDadec, bisnaphthalimidopropyl-diamino-decane; BNIPDpta, bisnaphthalimidopropyl-dipropyl-triamine; BNIPDeta, bisnaphthalimidopropyl-diethyl-triamine; DMF, dimethylformamide; THF, tetrahydrofuran; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Keywords: Bisnaphthalimidopropyl; Anticancer; Antiparasitic activity; Polyamines.

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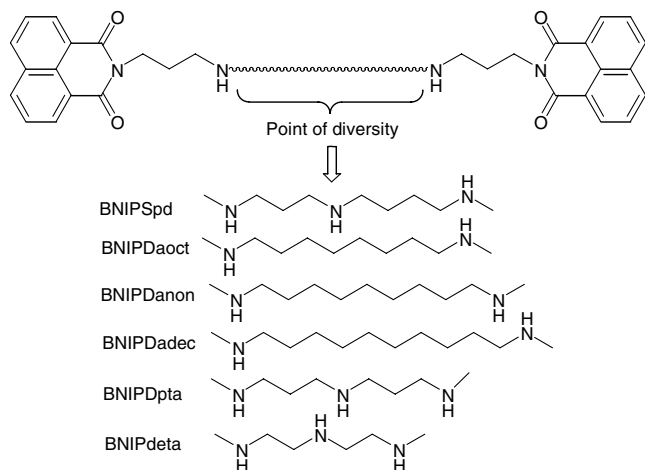


Figure 1. Bisnaphthalimidopropyl derivatives (BNIPSpd, BNIPDaact, BNIPDanon, BNIPDadec, BNIPDpta and BNIPDeta).

compounds fused with π excessive rings such as furan or thiophene.¹¹ In a short communication, we reported that HL60 promyelocytic leukaemia cells treated with bisnaphthalimidopropylspermidine (BNIPSpd) exhibited DNA fragmentation, elevated caspase-3 activity and form ‘condensed bodies’, suggesting strongly that the mechanism of cell death is by apoptosis.⁹ We also found for the first time that bisnaphthalimidopropyl derivatives exert significant antiproliferative effects on the life cycle of *Leishmania infantum*, the causative agent of visceral leishmaniasis. These drugs also induced the death of promastigotes by apoptosis.¹²

In this paper, we report the synthesis of analogues bisnaphthalimidopropyl di- and triamines, BNIPDaact, BNIPDanon, BNIPDadec, BNIPDpta and BNIPDeta, based on our lead compound BNIPSpd (spermidine derivative) with modification of the central chain as shown in Figure 1. The modification consists of different alkyl lengths of the central chain with 2 or 3 nitrogen atoms, thus modulating the number of positive charges in the molecules. We also discuss the in vitro cytotoxic properties of these newly synthesised compounds in colon cancer cells (Caco-2) and parasites (*L. infantum*, promastigotes).

2. Results and discussion

2.1. Chemistry

The synthetic strategy (Scheme 1) adopted to synthesise bisnaphthalimidopropyl derivatives BNIPSpd, BNIPDaact, BNIPDanon, BNIPDadec, BNIPDpta, and BNIPDeta was based on methods previously developed in our laboratory.^{3,10} Protection and activation of all the di- and triamines were carried out with mesitylene chloride in pyridine at room temperature to give compounds 1–6 in high yield. N-alkylation of the latter compounds with *O*-tosylpropylnaphthalimide 7 with caesium carbonate in anhydrous DMF afforded the fully protected bisnaphthalimidopropyl derivatives which upon deprotection with hydrobromic acid/glacial acetic acid in

CH_2Cl_2 gave BNIPSpd, BNIPDaact, BNIPDanon, BNIPDadec, BNIPDpta and BNIPDeta as their corresponding di- or trihydrobromide salts in yield varying from 50% to 70%.

2.2. Biological activities

The in vitro cytotoxicity of all the bisnaphthalimidopropyl derivatives described above was studied against colon cancer cell lines Caco-2 and parasite *L. infantum*. In the cancer cell line the IC_{50} values of each compound were determined after 24 and 48 h drug exposure (Table 1). All compounds except for BNIPDeta (IC_{50} values, 21.7 and 22.3 μM for 24 and 48 h, respectively), exerted IC_{50} values between 0.15 and 8.00 μM . BNIPSpd was the most active compound (IC_{50} , 0.47 and 0.15 μM at 24 and 48 h, respectively).

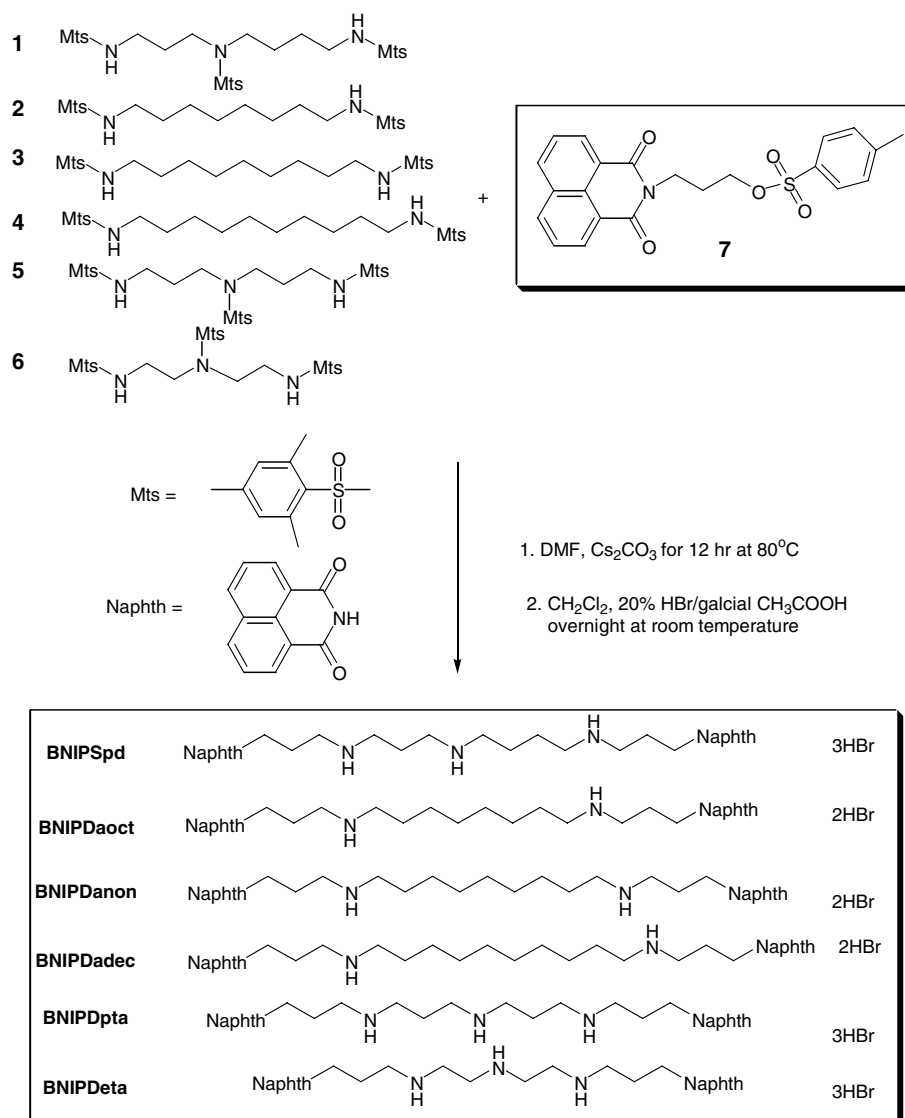
The removal of a nitrogen atom from the linker chain does not appear to substantially affect the cytotoxic properties of these compounds. We previously reported that when the central alkyl group is a butyl chain, the compound (BNIPPut) is not soluble in most solvents and the aqueous solubility of bisnaphthalimidopropyl compounds is enhanced by introducing a heteroatom like nitrogen in the central chain.³ Here, by increasing the length of the alkyl central chain such as in BNIPDaact, BNIPDanon and BNIPDadec also helps aqueous solubility. We reason that with the longer alkyl chain, the two naphthalimido rings do not tend to stack on top of each other by π – π interactions between the aromatic rings and hence favour aqueous solubility. Among the latter compounds, BNIPDadec showed the highest cytotoxicity against Caco-2 cells with IC_{50} values of 0.36 μM (48 h) and 0.77 μM (24 h).

Polyamine derivatives have recently shown promise in the search for more effective chemotherapeutic agents against parasite *L. infantum*. For example, N^4, N^8 -bis(3-phenylpropyl)spermine, N^4, N^8 -bis(3-naphthylmethyl)spermine and N^1, N^8 -bis(3-naphthylmethyl)spermidine were reported to be potent trypanocides in vitro with IC_{50} values ranging from 0.19 to 0.83 μM .¹³ More recently, using a combinatorial chemistry approach to produce a number of diamine libraries, Avery et al. reported a number of diamine derivatives with very good in vitro activity against *L. infantum* with the most active compound exhibiting an IC_{50} value of 0.88 μM .¹⁴ In our current series of bisnaphthalimidopropyl compounds we observed equally promising cytotoxic properties against parasite *L. infantum* (Table 2).

In conclusion, the new bisnaphthalimidopropyl derivatives exhibit cytotoxicity that may be further developed as antitumour and/or antiparasitic therapeutic agents. We are currently investigating the extent of DNA damage and repair in drug treated cells and these observations will be reported elsewhere.

2.3. Experimental

Caco-2 cells (ECACC, 86010202) were obtained from the European Collection of Cell Cultures. All reagents



Scheme 1. Synthetic strategy for the synthesis of bisnaphthalimidopropyl derivatives.

Table 1. Cytotoxicity of polyamine analogues against Caco-2 cancer cells

Compound ^a	IC ₅₀ (μM)	
	24 h	48 h
BNIPSpd	0.47 ± 0.12	0.15 ± 0.04
BNIPDaoct	6.20 ± 1.42	3.20 ± 0.67
BNIPDanon	3.60 ± 0.50	0.67 ± 0.11
BNIPDadec	0.77 ± 0.15	0.36 ± 0.08
BNIPDpta	5.14 ± 1.13	1.54 ± 0.51
BNIPDeta	21.7 ± 4.49	22.3 ± 5.62

^a Cytotoxicity determined by MTT assay. Data obtained after treating Caco-2 cells with varying concentrations of analogues (0.01–40 μM) for 24 and 48 h. Data are means ± SD of six replicates.

Table 2. Cytotoxicity of Polyamine derivatives against parasite *Leishmania infantum*

Compound ^a	IC ₅₀ (μM) 72 h
BNIPSpd	0.47 ± 0.01
BNIPDaoct	0.78 ± 0.049
BNIPDanon	0.78 ± 0.10
BNIPDadec	ND
BNIPDpta	1.54 ± 0.21
BNIPDeta	0.74 ± 0.11

^a Cytotoxicity determined by MTT assay. The results were obtained after treatment of the promastigote form of the parasite with different analogue concentrations (0.30–100 μM) after 72 h of incubation. The results are representative of medium ± SD at least five assays. ND, not determined.

were purchased from Aldrich, Fluka and Lancaster and were used without purification. TLC was performed on Kieselgel plates (Merck) 60 F₂₅₄ in chloroform: methanol (97:3 or 99:1). Column chromatography was done with silica gel 60, 230–400 meshes using chloroform

and methanol as eluent. FAB-mass spectra were obtained on a VG Analytical AutoSpec (25 kV) spectrometer, EC/CI spectra were performed on a Micromass Quattro II (low resolution) or on a VG Analytical

ZAB-E instrument (accurate mass). ^1H and ^{13}C NMR spectra were recorded on a JEOL JNM-EX90 FT NMR spectrometer.

BNIPSpd was synthesised according to our methods previously reported.^{3,10}

2.4. General method for the synthesis of mesitylated di- or triamine (1–6)

Corresponding diamine or triamine was dissolved in anhydrous pyridine followed by the addition of mesitylene chloride (2.1 M excess for diamine and 3.1 M excess for triamine). The resulting solution was stirred at room temperature for 4 h. Removal of the pyridine followed by the addition of cold water resulted in the formation of a precipitate. The latter was filtered off and washed thoroughly with water. The crude product was recrystallised from absolute ethanol.

2.4.1. N^1,N^8 -Dimesityloctane 2. (70%), ^{13}C NMR (CDCl_3) δ 20.82 (CH_3 , Mts), 22.85 (CH_3 , Mts), 26.34 (CH_2), 28.70 (CH_2), 29.41 (CH_2), 41.05 ($\text{N}-\text{CH}_2$), 47.58 (CH_2), 133 (aromatic carbons, Mts).

2.4.2. N^1,N^9 -Dimesitylnonane 3. (36%), ^{13}C NMR (CDCl_3) δ 20.82 (CH_3 , Mts), 22.85 (CH_3 , Mts), 26.34 (CH_2), 28.70 (CH_2), 29.41 (CH_2), 41.05 ($\text{N}-\text{CH}_2$), 47.58 (CH_2), 133 (aromatic carbons, Mts).

2.4.3. N^1,N^{10} -Dimesityldecane 4. (48%), ^{13}C NMR (CDCl_3) δ 20.82 (CH_3 , Mts), 22.85 (CH_3 , Mts), 26.34 (CH_2), 28.70 (CH_2), 29.41 (CH_2), 41.05 ($\text{N}-\text{CH}_2$), 47.58 (CH_2), 133 (aromatic carbons, Mts).

2.4.4. N^1,N^5,N^9 -Trimesityldipropyltriamine 5. (67%), ^{13}C NMR (CDCl_3) δ 20.85 (CH_3 , Mts), 22.79 (CH_3 , Mts), 27.69 (CH_2), 39.50 ($\text{N}-\text{CH}_2$), 43.11 ($\text{N}-\text{CH}_2$), 132.17 (aromatic carbons, Mts), 139.98 (aromatic carbons, Mts).

2.4.5. N^1,N^3,N^6 -Trimesityldiethyltriamine 6. (59%), ^{13}C NMR (CDCl_3) δ 21.35 (CH_3 , Mts), 23.06 (CH_3 , Mts), 41.05 ($\text{N}-\text{CH}_2$), 47.58 ($\text{N}-\text{CH}_2$), 133 (aromatic carbons, Mts).

2.5. Synthesis of *O*-tosylpropylnaphthalimide 7

Naphthalic anhydride (6.34 g, 0.032 mol) was dissolved in DMF (50 ml) followed by the addition of aminopropanol **3** (2.45 g, 0.032 mol) and DBU (4.87 g, 0.032 mol). The solution was left stirring at 85 °C for 4 h. The DMF was removed under reduced pressure and the resulting residue was poured into cold water with stirring (200 ml) to form a precipitate. The latter was filtered using a Buchner funnel and washed thoroughly with (i) water and (ii) saturated bicarbonate solution. The yield of the reaction was found to be 95%. This compound, naphthalimidopropanol, was pure enough and was used in the next step with no further purification. NMR (CDCl_3): δ 8.65–7.80 (m, 6H, aromatic protons), 4.39 (t, 2H, $-\text{N}-\text{CH}_2$), 3.69 (t, 2H, $\text{CH}_2-\text{O}-$), 3.20 (br s, 1H, OH), 2.06 (p, 2H, CH_2). ^{13}C NMR (CDCl_3): δ

161.70 ($\text{C}=\text{O}$), 135.70–122.90 (aromatic carbons), 74.90, 59.90, 30.90 ($3\times \text{CH}_2$).

Naphthalimidopropanol (5.10 g, 20 mmol) was dissolved in anhydrous pyridine (80 ml). The solution was stirred for 15 min at 0 °C. Tosyl chloride (5.72 g, 30 mmol) was added, in small portions, over 30 min. The solution was left overnight at 4 °C and was poured into ice water (200 ml) to form a solid on standing. The solid was filtered off and washed thoroughly with water. The crude product was recrystallised from either ethanol or ethyl acetate to give *O*-tosylpropylnaphthalimide **6** (53%). ^1H NMR (CDCl_3): δ 8.65–7.80 (m, 6H, aromatic protons), 4.45 (t, 2H, CH_2), 4.35 (t, 2H, CH_2), 2.50 (s, 3H, CH_3), 2.25 (p, 2H, CH_2). ^{13}C NMR (CDCl_3): δ 161.30 ($\text{C}=\text{O}$), 145.10–123.10 (aromatic carbons), 73.10, 67.90, 28.70 ($3\times \text{CH}_2$), 22.10 (CH_3). LRMS (FAB): Calcd for $\text{C}_{12}\text{H}_{19}\text{NO}_6\text{S}$ 425.09; found: 426 $[\text{MH}]^+$.

2.6. General N-alkylation reaction (step 1 in Scheme 1)

Mesitylated polyamines (**1–6**) (0.651 mmol) were dissolved in anhydrous DMF (13.5 ml) followed by the addition of **7** (0.13 mmol) and caesium carbonate (1.06 g). The solution was left at 80 °C. Completion of the reaction was monitored by thin-layer chromatography. DMF was removed under vacuo, and the residue was poured into cold water and the resulted precipitate filtered and washed thoroughly with water. After drying, the crude product was recrystallised from ethanol to give the fully protected pure product in high yield (75–85%).

2.7. General deprotection reaction (step 2 in Scheme 1)

The fully protected polyamine derivatives (0.222 mmol) were dissolved in anhydrous dichloromethane (10 ml) followed by the addition of hydrobromic acid/glacial acetic acid (1 ml). The solution was left stirring at room temperature for 24 h. The yellow precipitate formed was filtered off and washed with dichloromethane, ethyl acetate and ether.

2.7.1. BNIPSpd. (75%) $\text{DMSO}-d_6$, δ 22.20 (CH_2), 24.70 (CH_2), 44.10 ($\text{N}-\text{CH}_2$), 44.20 ($\text{N}-\text{CH}_2$), 45.00 ($\text{N}-\text{CH}_2$), 130 (aromatic carbons) 164.87 ($\text{C}=\text{O}$). LRMS (FAB): Calcd for $\text{C}_{37}\text{H}_{44}\text{N}_5\text{O}_4\text{Br}_3$ 862.1 $[\text{M}-3\text{HBr}]^+$ 619.3; found: 620.4 $[\text{M}-2\text{H}-3\text{Br}]^+$.

2.7.2. BNIPDaoct. (85%), $\text{DMSO}-d_6$, δ 24.43 (CH_2), 25.30 (CH_2), 25.66 (CH_2), 28.07 (CH_2), 44.72 ($\text{N}-\text{CH}_2$), 46.60 ($\text{N}-\text{CH}_2$), 121.99, 127.13, 130.62, 131.21, 134.31 (aromatic carbons), 163.61 ($\text{C}=\text{O}$). HRMS (FAB): Calcd for $\text{C}_{38}\text{H}_{44}\text{N}_4\text{O}_4 \text{Br}_2$ 778.1729, $[\text{M}-2\text{HBr}]^+$ 618.3206; found: 619.3282 $[\text{M}-\text{H}-2\text{Br}]^+$.

2.7.3. BNIPDanon. (85%), $\text{DMSO}-d_6$, δ 24.88 (CH_2), 25.84 (CH_2), 26.16 (CH_2), 28.76 (CH_2), 45.29 ($\text{N}-\text{CH}_2$), 47.29 ($\text{N}-\text{CH}_2$), 121.94, 127.51, 131.12, 131.42, 134.76 (naphthalimido aromatic carbons), 164.00 ($\text{C}=\text{O}$). HRMS (FAB): Calcd for $\text{C}_{39}\text{H}_{46}\text{N}_4\text{O}_4 \text{Br}_2$ 792.1886, $[\text{M}-2\text{HBr}]^+$ 632.3363, found: 633.3440 $[\text{M}-\text{H}-2\text{Br}]^+$.

2.7.4. BNIPDadec. (75%), DMSO- d_6 , δ 24.97 (CH_2), 25.90 (CH_2), 26.22 (CH_2), 28.79 (CH_2), 29.00 (CH_2), 45.35 (N- CH_2), 47.38 (N- CH_2), 121.05, 127.66, 131.30, 131.51, 134.94 (naphthalimido aromatic carbons), 164.21 (C=O). LRMS (FAB): Calcd for $\text{C}_{40}\text{H}_{48}\text{N}_4\text{O}_4 \text{ Br}_2$ 806.2, $([\text{M}-2\text{HBr}]^+)$ 646.4; found: 647.4 $[\text{M}-\text{H}-2\text{Br}]^+$.

2.7.5. BNIPDpta. (85%), DMSO- d_6 , δ 22.20 (CH_2), 24.70 (CH_2), 44.10 (N- CH_2), 44.20 (N- CH_2), 45.00 (N- CH_2), 130 (aromatic carbons) 164.87 (C=O). LRMS (FAB): Calcd for $\text{C}_{36}\text{H}_{42}\text{N}_5\text{O}_4 \text{ Br}_3$ 850.7, $([\text{M}-3\text{HBr}]^+)$ 605.3; found: 606.4 $[\text{M}-2\text{H}-3\text{Br}]^+$.

2.7.6. BNIPDeta. (67%), DMSO- d_6 , δ 22.20 (CH_2), 24.70 (CH_2), 44.10 (N- CH_2), 44.20 (N- CH_2), 45.00 (N- CH_2), 130 (aromatic carbons). HRMS (FAB): Calcd for $\text{C}_{34}\text{H}_{38}\text{N}_5\text{O}_4 \text{ Br}_3$ 817.0474, $([\text{M}-3\text{HBr}]^+)$ 577.2689; found: 578.2760 $[\text{M}-2\text{H}-3\text{Br}]^+$.

2.8. Cytotoxic studies

Cytotoxicity was evaluated for Caco-2 colon carcinoma and *L. infantum* using the MTT assay with protocols appropriate for the individual test system.^{10,12} Caco-2 cells were maintained in Earle's Minimum Essential Medium (Sigma), supplemented with 10% foetal calf serum (Biosera), 2 M L-glutamine (Sigma), 1% non-essential amino acids (Sigma), 100 IU/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (Sigma). Exponentially growing cells were plated at 2×10^4 cells cm^{-2} into 96-well plates and incubated for 24 h before the addition of drugs. Stock solutions of compounds were initially dissolved in 20% DMSO and further diluted with fresh complete medium.

After 24 and 48 h of incubation at 37 °C, the medium was removed and 200 μl of MTT reagent (1 mg/ml) in serum-free medium was added to each well. The plates were incubated at 37 °C for 4 h. At the end of the incubation period, the medium was removed and pure DMSO (200 μl) was added to each well. The metabolized MTT product dissolved in DMSO was quantified by reading the absorbance at 560 nm on a micro plate reader (Dynex Technologies, USA). IC_{50} values are defined, as the drug concentrations required to reduce the absorbance by 50% of the control values. The IC_{50} values were calculated from the equation of the logarithmic line determined by fitting the best line (Microsoft Excel) to the curve formed from the data. The IC_{50} value was obtained from the equation for $y = 50$ (50% value).

Leishmania infantum (clone MHOM/MA671TMA-P263) promastigotes were grown at 27 °C in RPMI medium (Gibco) supplemented with 10% of heat-inactivated foetal bovine serum (FBS-Gibco), 2 mM L-glutamine (Gibco), 20 mM Hepes (Gibco), 100 U/ml penicillin (Gibco) and 100 $\mu\text{g}/\text{ml}$ streptomycin (Gibco). The parasites ($10^6/\text{ml}$) in the logarithmic phase (2 days

of culture) were incubated with a serial range of concentrations of each drug for 3 days at 27 °C and the growth of parasites was determined by the MTT assay. Briefly, the MTT solution was added from a 5 mg/ml stock solution to have 0.25 mg/ml in the wells. The plates were incubated at 27 °C for 4 h. At the end of the incubation period 50 μl of a solution containing 20% of SDS, 50% of DMF and pH of 4.8 was added. After 1 h at 37 °C the absorbance was read at 550 nm on a micro plate reader. The IC_{50} is the concentration of the drug required to inhibit the growth by 50% was determined by linear regression analysis.

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2.7 Bisnaphthalimidopropyl derivative compounds as novel *Leishmania* SIR2RP1 inhibitors

The NAD⁺-dependent deacetylases, namely sirtuins, have been involved in the regulation of a number of biological processes, such as gene silencing, DNA repair, longevity, metabolism, apoptosis, and development. A *Leishmania infantum* protein belonging to this family, LiSIR2RP1, is a tubulin NAD⁺-dependent deacetylase and an ADP-ribosyltransferase. The involvement of this protein in the parasite's virulence and survival disclosed its potential as a drug target. Our search for selective inhibitors of LiSIR2RP1 has led, for the first time, to the identification of the antiparasitic and anticancer bisnaphthalimidopropyl (BNIP) alkyl di- and tri- amines as inhibitors of LiSIR2RP1 (IC₅₀ in the single μ M range for the most potent compounds). Structure-activity studies were conducted with 12 BNIP derivatives that differed in the length of the alkyl central chain which linked the two naphthalimidopropyl moieties. The most active and selective compound was the BNIP diaminononane (BNIPDanon), with IC₅₀ values of 5.7 and 97.4 μ M against the parasite and the human SIRT1 enzyme, respectively. Furthermore, this compound is an NAD⁺ competitive inhibitor that interacts differently with the parasite and human enzyme, as determined by docking analysis, thus explaining its selectivity towards the parasitic enzyme.

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Bisnaphthalimidopropyl derivative compounds as novel *Leishmania* SIR2RP1 inhibitors

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Abstract

The NAD⁺-dependent deacetylases, namely sirtuins, have been involved in the regulation of a number of biological processes, such as gene silencing, DNA repair, longevity, metabolism, apoptosis, and development. A *Leishmania infantum* protein belonging to this family, LiSIR2RP1, is a tubulin NAD⁺-dependent deacetylase and an ADP-ribosyltransferase. The involvement of this protein in the parasite's virulence and survival disclosed its potential as a drug target. Our search for selective inhibitors of LiSIR2RP1 has led, for the first time, to the identification of the antiparasitic and anticancer bisnaphthalimidopropyl (BNIP) alkyl di- and tri- amines as inhibitors of LiSIR2RP1 (IC₅₀ in the single µM range for the most potent compounds). Structure-activity studies were conducted with 12 BNIP derivatives that differed in the length of the alkyl central chain which linked the two naphthalimidopropyl moieties. The most active and selective compound was the BNIP diaminononane (BNIPDanon), with IC₅₀ values of 5.7 and 97.4µM against the parasite and the human SIRT1 enzyme, respectively. Furthermore, this compound is an NAD⁺ competitive inhibitor that interacts differently with the parasite and human enzyme, as determined by docking analysis, thus explaining its selectivity towards the parasitic enzyme.

Introduction

The proteins belonging to the Silent Information Regulator 2 (SIR2) family, also known as sirtuins, have been involved in the regulation of a number of biological processes, such as heterochromatin formation, gene silencing, DNA repair, development, longevity, metabolism, adipogenesis, and apoptosis (reviewed in Zhao and Marmorstein, 2006). Indeed, these proteins are classified as class III histone deacetylases, due to their dependency on NAD^+ to deacetylate lysine residues of histones and non-histone substrates (Imai et al., 2000; North et al., 2003; Starai et al., 2002). Besides the generation of the deacetylated substrate, nicotinamide and O-acetyl-ADP-ribose (OAADPr) are also products of the reactions catalyzed by sirtuins (Tanny and Moazed, 2001; Sauve et al., 2001). Apart from the deacetylase activity, some sirtuins also exhibit ADP-ribosyltransferase activity (Sauve and Schramm, 2004; Liszt et al., 2005).

This family of proteins is present in a variety of organisms ranging from bacteria to humans, (Brachmann et al., 1995) and the presence of a conserved catalytic core domain with variable N and C terminal extensions is a hallmark of all the resolved sirtuin crystal X-ray structures (Avalos et al. 2005; Finnin et al., 2001). The catalytic core domain is constituted by a large classical Rossmann fold and a small zinc-binding domain. The interface between the large and the small domain is commonly divided into A, B and C pocket. This designation is based on the interaction of the adenine (A), ribose (B), and nicotinamide (C) that constitutes the NAD^+ cofactor. While the class I and II histone deacetylases had already been validated as anticancer drug targets with some of the inhibitors having reached clinical trials, less is known about the inhibition of the class III members (Johnstone et al., 2002). However, recent findings suggest a direct link between the activity of sirtuins and diseases such as cancer, HIV and Parkinson (Pagans et al., 2005; Vaziri et al., 2001; Bereshchenko et al., 2002; Ota et al., 2006; Outeiro et al., 2007). The first reports on Sirtuin inhibitors identified, beyond the physiological inhibitor nicotinamide (Fig 1, A), sirtinol (Fig 1, B) and splitomicin (Fig 1, C) (Grozing et al., 2001; Bedalov et al., 2001). Since they all exhibited weak inhibitory properties, subsequent structure-activity studies have led to the identification of more potent derivatives, such as HR73 and β -phenylsplitomicin (Mai et al., 2005; Pagans et al., 2005; Neugebauer et al., 2008). Furthermore, a high-throughput screening against the human sirtuin, SIRT1, has led to the identification of indoles (Fig 1, D) as the most potent ($\text{IC}_{50} < 0.1 \mu\text{M}$) and selective inhibitors described to date (Napper et al., 2005). In addition, drugs that mimic adenosine (suramin, Fig 1, E) or target enzymes or receptors, like kinases, that bind to adenosine-containing cofactors or

ligands were identified as human SIR2 inhibitors (Howitz et al., 2003; Trapp et al., 2007). Moreover, in a recent study, the crystal structure of SIRT5 bound to suramin allowed to solve the nature of SIRT5's enzymatic inhibition at the molecular level (Schuetz et al., 2007)

Leishmaniasis is a parasitic disease caused by the protozoan parasites of the genus *Leishmania*, and is characterized by diverse clinical manifestations, varying from localized ulcerative skin lesions to disseminated visceral infection. The latter is fatal when left untreated. *Leishmania* infects a vertebrate host after the bite of a sandfly (*Phlebotomus* and *Lutzomyia* spp.) during a blood meal, through the inoculation of infective flagellated promastigotes that invade or are phagocytosed by local or recruited host cells. In the phagolysosomes, the promastigotes will differentiate into non-flagellated amastigotes that multiply and are able to infect other adjacent or distant macrophages. The classical treatment is currently unsatisfactory due to side effects, the emergence of resistances and the need for increased efficacy in immunosuppressed patients, especially due to HIV co-infection. During the last few years, we have been interested in the protozoan parasite *Leishmania infantum* SIR2-related protein 1, LiSIR2RP1, due to its role in the parasite's survival and virulence (Vergnes et al., 2002; 2005). Indeed, this cytosolic parasite protein, partially associated with the microtubule network, is a tubulin NAD⁺ dependent deacetylase and ADP-ribosyltransferase (Tavares et al., 2008).

In previous studies, we have shown that bisnaphthalimidopropyl (BNIP) polyamine derivatives exerted anti-*Leishmania* activity *in vitro*, inducing the death of promastigote forms by apoptosis (Tavares et al., 2005). The search for target specific SIR2 inhibitors led us to evaluate whether BNIP derivatives could selectively inhibit the parasitic enzyme (Kong Thoo Lin and Pavlov, 2000; Tavares et al., 2005; Oliveira et al., 2007).

In the present study, we report for the first time a structure-activity study using 12 BNIP derivative compounds, the human SIRT1 and the LiSIR2RP1. The compounds differed in the alkyl length of the central chain with 2, 3 or 4 N atoms, linking the 2 naphthalimidopropyl groups. We found that the most potent and selective inhibitor of the parasitic enzyme (LiSIR2RP1) was the bisnaphthalimidopropylidiaminononane (BNIPDanon), that contains an alkyl linker chain with 9 carbon and 2 nitrogen atoms. Furthermore, this compound is an NAD⁺ competitive inhibitor, and the molecular docking analysis revealed that its different interference with the parasite and human enzyme explains the selectivity towards the parasitic enzyme.

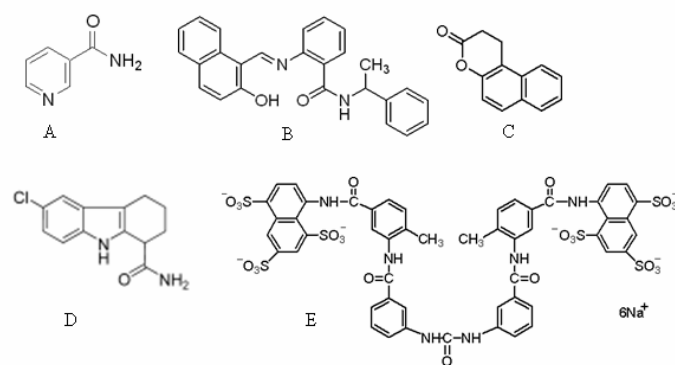


Figure 1. Examples of NAD⁺ dependent SIR2 inhibitors. Nicotinamide (A), Sirtinol (B), Splitomicin (C), an Indole derivative (D) and Suramin (E).

Experimental Section

Chemistry

All reagents for the synthesis were from Aldrich-Sigma and Fluka and were used without purification. TLC was performed on Kieselgel plates (Merck) 60 F₂₅₄ in chloroform: methanol (97:3 or 99:1). FAB-mass spectra were obtained on a VG Analytical AutoSpec (25Kv) spectrometer, EC/CI spectra were performed on a Micromass Quattro II (low resolution) or on a VG Analytical ZAB-E instrument (accurate mass). ¹H and ¹³C NMR spectra were recorded on a JEOL JNM-EX90 FT and Bruker 400 MHz NMR spectrometers.

All compounds used in this work, with the exception of bisnaphthalimidopropyldiamino: pentane (BNIPDapen), hexane (BNIPDahehex), heptane (BNIPDahep) and dodecane (BNIPDadodec), had previously been described (Kong Thoo Lin and Pavlov, 2000; Oliveira et al., 2007). The synthesis of the former compounds is described below.

Step 1

Corresponding Diamino- pentane, hexane, heptane and dodecane were dissolved in anhydrous pyridine, followed by the addition of mesitylene chloride (2.1 molar excess). The resulting solution was stirred at room temperature for 4 hours. Removal of the pyridine followed by the addition of cold water resulted in the formation of a precipitate. The latter was filtered off and washed thoroughly with water. The crude product was recrystallized from absolute ethanol.

Step 2

Mesitylated diamines (0.651mmol) were dissolved in anhydrous DMF (13.5ml), followed by the addition of O-tosylpropylphthalimide (Oliveira et al., 2007) (0.13mmol) and cesium carbonate (1.06g). The solution was left overnight at 80°C. Completion of the reaction was monitored by thin layer chromatography. DMF was removed under *vacuo*, and the residue was poured into cold water and the resulted precipitate filtered and washed thoroughly with water. After drying, the crude product was recrystallized from ethanol to give the fully protected pure product in high yield (75-85%).

Step 3

The fully protected polyamine derivatives (0.222mmol) were dissolved in anhydrous dichloromethane (10ml), followed by the addition of hydrobromic acid/glacial acetic acid (1ml). The solution was left stirring at room temperature for 24h. The yellow precipitate formed was filtered off and washed with dichloromethane, ethylacetate and ether.

BNIPDapen (83%), DMSO- d_6 , δ : 22.91 (2xCH₂), 24.46 (CH₂), 24.79 (CH₂), 36.70 (CH₂), 44.72 (N-CH₂), 46.27 (N-CH₂), 121.99, 127.13, 130.62, 131.21, 134.31 (Aromatic Carbons), 163.61 (C=O). LRMS (FAB): Calcd. for C₃₅H₃₈N₄O₄ Br₂ 738.520, found: 657.1 [M-HBr]⁺, 577.2 ([M-2HBr]⁺

BNIPDahehex (91%), DMSO- d_6 , δ : 24.52 (CH₂), 25.21 (CH₂), 25.39 (CH₂), 36.72 (CH₂), 44.81 (N-CH₂), 46.54 (N-CH₂), 121.99, 127.13, 130.62, 131.21, 134.31 (Aromatic Carbons), 163.61 (C=O). HRMS (FAB): Calcd. for C₃₆H₄₀N₄O₄ Br₂ 752.510, 671.2227 [M-Br]⁺, found: 671.2221 [M-Br]⁺.

BNIPDahep (94%), DMSO- d_6 , δ : 24.46 (CH₂), 25.30 (CH₂), 25.66 (CH₂), 27.84, (CH₂), 36.70 (CH₂), 44.72 (N-CH₂), 46.60 (N-CH₂), 121.99, 127.13, 130.62, 131.21, 134.31 (Aromatic Carbons), 163.58 (C=O). HRMS (FAB): Calcd. for C₃₇H₄₂N₄O₄ Br₂ 760.74, 605.3122 [M-2HBr+H]⁺, found: 605.3126 [M-2HBr+H]⁺.

BNIPDadodec (80%), DMSO- d_6 , δ : 24.46 (CH₂), 25.39 (CH₂), 25.84 (CH₂), 28.40 (CH₂), 28.73 (CH₂), 28.82, (CH₂), 36.70 (CH₂), 44.72 (N-CH₂), 46.60 (N-CH₂), 121.99, 127.13, 130.62, 131.21, 134.31 (Aromatic Carbons), 163.64 (C=O). HRMS (FAB): Calcd. for C₄₂H₅₂N₄O₄ Br₂ 836.709, 755.3166 [M-Br]⁺, found: 755.3168 [M-Br]⁺.

Compounds

Sirtinol, nicotinamide, and suramin were purchased from Sigma. Stock solutions of nicotinamide and suramin were prepared in phosphate saline buffer (PBS) and the other compounds in DMSO, and they were all stored at -20°C. Working solutions were freshly diluted in the enzymatic reaction buffer until the desired final concentrations.

Fluorimetric deacetylase assay of recombinant LiSIR2RP1 and human SIRT1

The LiSIR2RP1 (N-terminal His6-tag) was expressed in *E coli* and purified by affinity chromatography, as already reported by our group (Tavares et al., 2008). The effect of the several compounds in the NAD⁺-dependent deacetylase activity of the parasite (LiSIR2RP1) and the human enzyme (SIRT1) was assayed by using a commercially available CycLex SIRT1/Sir2 deacetylase fluorometric kit (Cyclex Co., Ltd., Nagano, Japan). This assay system allows the detection of a fluorescent signal upon deacetylation of the peptide substrate, followed by cleavage through the action of a protease. Fluorescence was measured in a fluorometric microplate reader (Synergy HT; BIO-TEK) with excitation and emission wavelengths set at 340nm and 440nm, respectively. Reactions were performed in the presence of 200μM of NAD⁺, 10μM of acetylated peptide and each of the inhibitors at a range of several concentrations.

The inhibitory effect of the drugs in the rLiSIR2RP1 and hSIRT1 activity is expressed in percentage, and was calculated according to the ratio between the rates of the enzymatic reaction in the first 20 minutes in the presence and absence of the inhibitor.

Tubulin deacetylation assay

The deacetylation reactions where tubulin was used as a substrate were performed using purified tubulin (Pure, Cytoskeleton Inc). The reactions were carried out in deacetylase buffer (10mM Tris-HCl, pH 8.0 and 10mM NaCl), containing 0.5μg of either tubulin and LiSIR2RP1, in the presence or absence of 1mM NAD⁺ and left overnight with constant agitation at room temperature (RT). The inhibitors, BNIPDadec and nicotinamide, were tested at the following concentrations: 0, 0.125, 0.250 and 0.5mM. The amount of DMSO present in the BNIPDadec tested concentrations was included as a control. The reactions were stopped by adding 5x Laemmli sample buffer, and the proteins separated by 10% SDS-PAGE and western blotted. The nitrocellulose membrane was probed with the following antibodies: mouse monoclonal anti-acetylated tubulin antibody (clone 6-11B-1) from Sigma;

mouse monoclonal anti- α -tubulin antibody (clone DM1A) from NeoMarkers, and the mouse monoclonal antibody, IIIG4 produced as described by Vergnes et al., 2002.

Modelling and docking of inhibitors

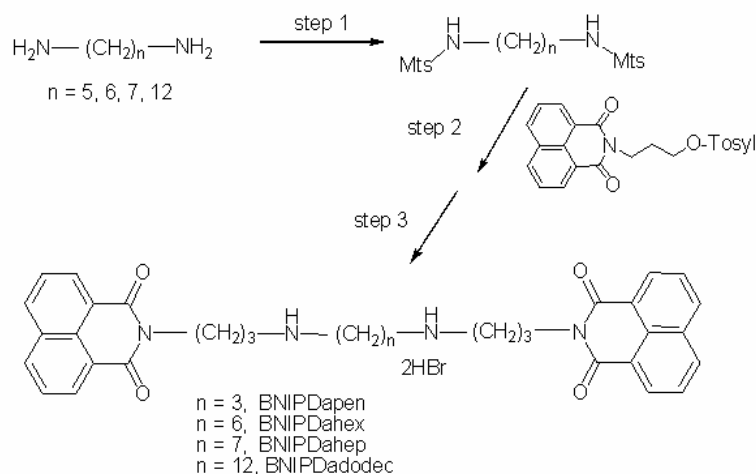
Homology modelling was done for hSIRT1 and LiSIR2RP1 using the comparative protein modelling program MODELLER. The crystal structure of human SIRT2 (PDB ID-1j8f) was used as a template in both cases. Sequences were aligned using the align2d command in MODELLER. The optimization of the models was done using molecular dynamics (MD) with the simulated annealing (SA) method in MODELLER. Out of the five models generated for each protein, the best model was evaluated based on the lowest MODELLER Objective function. The quality and geometry of models was checked using the program PROCHECK and VERIFY_3D in SAVES server. The NAD molecule was added to the models using MOE. These models were then subjected to further refinement and energy minimization using the Biopolymer module in Sybyl. Molecular surface was generated using the Molcad module in Sybyl to analyze and compare the binding pocket of NAD in both structures. Four BNIP derivatives, BNIPDahehex, BNIPDanon, BNIPDadec and BNIPDadodec, showing variability in IC₅₀ values, were selected to perform docking for LiSIR2RP1 and hSIRT1 using the FlexX module in Sybyl.

Results and Discussion

Chemistry

The synthetic strategy (Scheme 1) adopted to synthesise the bisnaphthalimidopropyl derivatives, BNIPDapen, BNIPDahehex, BNIPDahep and BNIPDadodec, was based on methods previously developed by our group (Lin and Pavlov, 2000; Dance, et al., 2005; Oliveira et al., 2007).

Essentially, the synthesis involves a 3-step reaction (Scheme 1). Penta-, hexa-, hepta- and dodeca- diamines were first mesitylated with mesitylchloride in pyridine at room temperature. N-alkylation between the N-mesitylatedalkyl diamines and o-tosylpropylnaphthalimide (Oliveira et al., 2007) gave the corresponding protected products, which upon treatment with hydrobromic/glacial acetic acid yielded the products in high yield (80-94%).



Scheme 1. Step 1, Mts-Cl/pyridine at RT; Step 2, DMF/Cs₂CO₃ at 85°C for 12 hr; Step 3, HBr/glacial Acetic Acid in CH₂Cl₂ at RT for 12 hr.

Enzyme inhibition

All of the BNIP derivative compounds and some of the commercially available Sirtuin inhibitors, such as nicotinamide, sirtinol and suramin were tested *in vitro* for their ability to inhibit the *L. infantum* SIR2RP1 and the human SIRT1. The inhibition experiments were conducted using a commercially available fluorimetric deacetylase assay. This double enzymatic assay uses a peptide containing an acetylated lysine as substrate. Once the peptide is deacetylated by sirtuins, it becomes substrate to a lysilendopeptidase that allows the release of a fluorescent metabolite. To disclose that any of the inhibitory activity observed for the tested compounds was not due to an inhibition of the lysilendopeptidase, we have tested the effect of each compound in this enzyme. This was achieved by using an already deacetylated peptide instead of the acetylated one. None of the compounds was able to inhibit the lysilendopeptidase, disclosing that any inhibition is due to their interference with the NAD⁺-dependent deacetylase activity of LiSIR2RP1 or hSIRT1 (data not shown).

We have recently demonstrated that LiSIR2RP1 is an ADP-ribosyltransferase and NAD⁺-dependent deacetylase which could be inhibited in a non-competitive manner by nicotinamide (Tavares et al., 2008). The mechanism by which nicotinamide inhibits the deacetylation activity of Sirtuins is already known. In fact, nicotinamide inhibits these enzymes by interacting with a reaction intermediate. The positively charged O-alkyl-amidate intermediate and nicotinamide are generated after the nucleophilic attack of the acetyl-lysine carbonyl oxygen on the C1' to the

nicotinamide ribose of NAD^+ (Denu et al., 2003; Sauve et al., 2001; Sauve and Schramm, 2004). When nicotinamide binds to the enzyme containing the O-alkyl-amidate intermediate, both can react in a process known as nicotinamide exchange, where the NAD^+ and the acetylated peptide are reformed (Jackson et al., 2003; Sauve et al., 2001; Sauve and Schramm, 2003). In the presence of high concentrations of nicotinamide, this reaction occurs at the expense of deacetylation. The results in Table 1 indicate that LiSIR2RP1 is more sensitive to nicotinamide inhibition (IC_{50} $39.4 \pm 5.0 \mu\text{M}$) than hSIRT1 (IC_{50} $118.3 \pm 23.6 \mu\text{M}$). This could be due to differences on the flexible loop of the respective enzymes (that seem to be involved in the recognition of different substrates) and its close contact with the C pocket. Indeed, Avalos et al. (2005) disclosed the mechanism of Sirtuins' inhibition by nicotinamide, highlighting the role of the C pocket. A structure-based mechanism, where nicotinamide could exist in either, a reactive or entrapped conformation, and the O-alkyl-amidate intermediate that could exist in a contracted or extended conformation seem to be key factors for the occurrence of the deacetylation or the nicotinamide exchange reaction. Furthermore, different sensitivities to nicotinamide inhibition were reported about the yeast SIR2, when complexed with different proteins (Tanny et al., 2004).

Sirtinol has low inhibitory potency on the parasite enzyme with an IC_{50} of $193.8 \pm 31.8 \mu\text{M}$ (Table 1). The IC_{50} on the hSIRT1 that was determined in this study ($245.5 \pm 3.5 \mu\text{M}$, Table 1) is higher than the one reported by others (Mai A. et al., 2005). This could be due to its low solubility in aqueous solutions, although no significative differences concerning the sirtinol potency were observed between the parasite and the human enzymes.

Suramin is a symmetric polyanionic naphthylurea originally used to treat sleeping sickness and onchocerciasis. Several other biological functions have been attributed to this compound and its derivatives, such as antiproliferative and antiviral activities (Voogd et al., 1993). Suramin was found to be an inhibitor of the human NAD^+ -dependent deacetylases, namely SIRT1, SIRT2 and SIRT5 (Schuetz et al., 2007; Trapp et al., 2007). In this study, we showed that suramin is a more active inhibitor of the hSIRT1 (IC_{50} value of $2.4 \pm 0.5 \mu\text{M}$) than the parasite enzyme (IC_{50} $6.8 \pm 0.7 \mu\text{M}$) (Table 1). The inhibitory activity towards the hSIRT1 is in accordance with the results reported by Schuetz and colleagues (2007). Furthermore, the structural basis of the hSIRT5 inhibition by suramin revealed that this molecule interacts with the B- and C- pockets of the NAD^+ binding site as well as with the substrate-binding site. Additionally, suramin acts as a linker molecule leading to the dimerization of hSIRT5 (Schuetz et al., 2007). A similar mechanism of inhibition is

suggested by molecular docking and analysis of favorable interactions to the human SIRT1 and SIRT2 (Trapp et al., 2007).

Table 1. *L. infantum* SIR2RP1 (LiSIR2RP1) and human SIRT1 (hSIRT1) inhibitory activity of known sirtuin inhibitors.

Compound name	IC ₅₀ ± SD (μM)		Potency index (LiSIR2RP1/hSIRT1)
	LiSIR2RP1	hSIRT1	
Nicotinamide	39.4 ± 5.0	118.3 ± 23.6	3.00
Sirtinol	193.8 ± 31.8	245.5 ± 3.5	1.28
Suramine	6.8 ± 0.7	2.4 ± 0.5	0.35

IC₅₀ (Concentration of drug that inhibits 50% of the enzymatic activity pertaining to the control) ± SD (Standard Deviation) data are reported as the mean of at least three independent experiments.

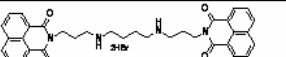
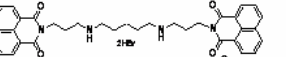
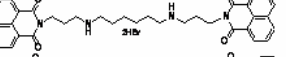
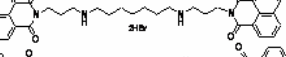
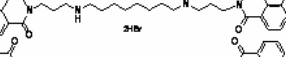
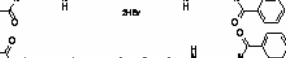
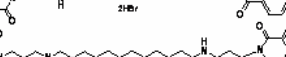
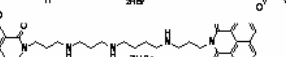
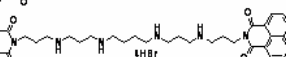
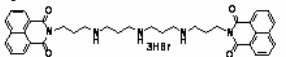
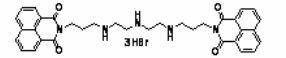
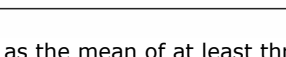
A series of bisnaphthalimidopropyl diamine derivatives, containing an alkyl linker chain with 4 (compound 1) to 11 (compound 8) carbon atoms, were synthesized. We reasoned that by increasing the length of the alkyl chain, the two naphthalimido rings do not tend to stack on top of each other by π - π interactions between the aromatic rings. The effect of introducing positive charges, through nitrogen atoms, on the bisnaphthalimido linker chain was also evaluated using the compounds 9 to 12. All of the tested bisnaphthalimidopropyl derivatives were capable of inhibiting the NAD⁺-dependent deacetylase activity of LiSIR2RP1 with IC₅₀ values lower than 55 μM. The most active compound against this enzyme (BNIPDanon, 6) exhibited an IC₅₀ in the single micromolar range of 5.7 ± 0.2 μM (Table 2). On the other hand, the less effective compound was the BNIPDeta (12) with an IC₅₀ of 54.7 ± 15.7 μM (Table 2). Considering the potency index, which is the efficiency of the BNIP derivatives to inhibit LiSIR2RP1 when compared to hSIRT1, it seems to be dependent on the length and charge of the naphthalimidopropylamine group's linker chain. Indeed, BNIP diamine derivatives containing between 4 to 7 carbon atoms (compounds 1 to 4) on the linker chain were less active than the ones containing between 8 to 12 carbon atoms (compounds 5 to 8). The optimal distance between the two naphthalimidopropylamine groups that results in the most effective inhibitory activity of LiSIR2RP1 was obtained with the compound containing 9 carbon atoms (BNIPDanon, 6). The introduction of positive charges in the linker chain does not improve the compounds' potency. As an example, the

BNIPSpd (compound 9) has 8 atoms in the naphthalimidopropylamine linker chain, of which 7 are carbon and 1 is a nitrogen atom, and it is less active (IC_{50} $17.9 \pm 1.6\mu M$) than the BNIPDaoct (5) that contains 8 carbon atoms (IC_{50} $9.2 \pm 1.4\mu M$) (Table 2).

All of the BNIP derivatives showed to be less potent inhibitors against the hSIRT1 than towards the parasite enzyme. The IC_{50} values obtained when using hSIRT1 vary between 43.1 to $182.8\mu M$ (Table 2).

In contrast to what was observed with LiSIR2RP1, the changes in the linker chain do not significantly affect the potency of compounds to inhibit hSIRT1. Indeed, the most active compound on this enzyme was the BNIPDeta (12) with an of IC_{50} $43.1 \pm 9.3\mu M$. Therefore, these results suggest a certain degree of selectivity of some BNIP derivatives towards the parasite enzyme. The highest selectivity was obtained with the BNIPDanon (6), followed by the BNIPDaoct (5) and the BNIPDadec (7), since they were respectively 17, 12.7 or 10.2 times more active against the parasite than the hSIRT1.

Table 2. *L. infantum* SIR2RP1 (LiSIR2RP1) and human SIRT1 (hSIRT1) inhibitory activity of bisnaphthalimidopropyl (BNIP) derivatives.

Compound	Name	Structure	$IC_{50} \pm SD (\mu M)$		Potency index (LiSIR2RP1/hSIRT1)
			LiSIR2RP1	hSIRT1	
1	BNIPDabut		35.0 ± 5.8	73.1 ± 14.9	2.1
2	BNIPDapen		37.7 ± 6.9	82.2 ± 16.4	2.2
3	BNIPDahex		43.3 ± 9.5	93.5 ± 7.8	2.2
4	BNIPDahep		52.7 ± 5.2	127.5 ± 31.9	2.4
5	BNIPDaoct		9.2 ± 1.4	116.5 ± 23.3	12.7
6	BNIPDanon		5.7 ± 0.2	97.4 ± 4.9	17.0
7	BNIPDadec		11.2 ± 1.6	113.8 ± 22.7	10.2
8	BNIPDadodec		10.1 ± 1.2	94.7 ± 23.7	9.4
9	BNIPSpd		17.9 ± 1.6	94.8 ± 23.7	5.3
10	BNIPSpm		39.5 ± 6.5	102.2 ± 31.9	2.6
11	BNIPDpta		32.8 ± 2.4	43.1 ± 9.3	1.3
12	BNIPDeta		54.7 ± 15.7	182.8 ± 22.2	3.3

$IC_{50} \pm SD$ data are reported as the mean of at least three independent experiments.

Complementary experiments were done using an alternative method to evaluate the inhibitory effect of the BNIP derivatives on the NAD⁺-dependent deacetylase activity of LiSIR2RP1 (Howitz et al., 2003; Kaeberlein et al., 2005; Borra et al., 2005). Indeed, we have shown that LiSIR2RP1 in the presence of NAD⁺ deacetylates purified tubulin. The reaction could be inhibited by nicotinamide and visualized by Western Blot using specific antibodies to either total α -tubulin or its acetylated form (Tavares et al., 2008). Figure 2 shows the effect of increasing concentrations of nicotinamide (0.125, 0.25 and 0.5mM) or BNIPDadec (0.125, 0.25 and 0.5mM) on the NAD⁺-dependent deacetylation of tubulin (0.5 μ g) mediated by rLiSIR2RP1 (0.5 μ g). Since DMSO was used to dissolve the BNIPDadec, increasing amounts of DMSO corresponding to the concentration present in each test tube were used as controls. As expected, none of the DMSO concentration could inhibit tubulin deacetylation by LiSIR2RP1. In contrast, increasing amounts of BNIPDadec or nicotinamide were capable of inhibiting the tubulin deacetylation mediated by LiSIR2RP1.

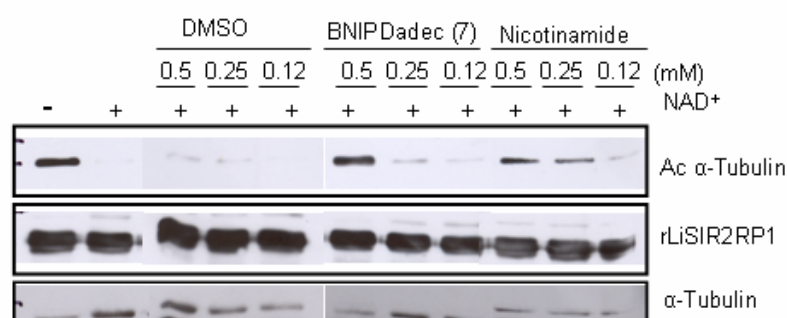


Figure 2. BNIPDadec (7) inhibits the deacetylation of α -tubulin by LiSIR2RP1. Purified tubulin dimmers were incubated overnight at room temperature with purified rLiSIR2RP1 in the presence or absence of 1mM of NAD⁺ and 0.5, 0.25, 0.125 or 0 mM of nicotinamide, BNIPDadec (7) and DMSO (equivalent amount to the one present in each BNIPDadec concentration). The reaction products were analyzed by western blotting with specific antibodies to acetylated α -tubulin, α -tubulin, and LiSIR2RP1.

To further analyse the BNIP inhibitory activity, kinetic studies were performed with rLiSIR2RP1 and its most active inhibitor, BNIPDanon (6), by combining different drug and NAD⁺ concentrations (Fig.3). A similar approach was conducted using increasing amounts of acetylated peptide substrate; the results are shown as Lineweaver-Burk plots (Fig 3). BNIPDanon (6) did not act as a competitive inhibitor

of the LiSIR2RP1 substrate (Fig 3A). Its inhibitory effect on the LiSIR2RP1 deacetylase activity is due to competition with the NAD^+ (Fig 3B). The K_M values obtained for NAD^+ differed in the presence of 0, 3 or $6\mu\text{M}$ of BNIPDanon and were respectively, 45.0, 78.14, and $149.9\mu\text{M}$, while no significative changes were obtained between their respective $V_{\max}$ values (138.9 , 140.8 , and $112.4 \text{ Fluores}_{340/440} \cdot \text{min}^{-1}$). Based on these data, we can hypothesize that BNIPDanon interacts with the NAD^+ binding site of the enzyme, or at least close enough to induce structural changes that interfere with it binding.

Suramin and other related adenosine receptor antagonists, like kinase inhibitors, were reported to be sirtuin inhibitors (Howitz et al., 2003; Trapp et al., 2006; Schuetz et al., 2007; Trapp et al., 2007). In fact, the screening of a library of kinase inhibitors led to the identification of a disubstituted Bis(indol)maleimide as a potent (IC_{50} values of 3.5 and $0.8\mu\text{M}$ against the hSIRT1 and the hSIRT2 respectively) and competitive inhibitor with NAD^+ (Trapp et al., 2007).

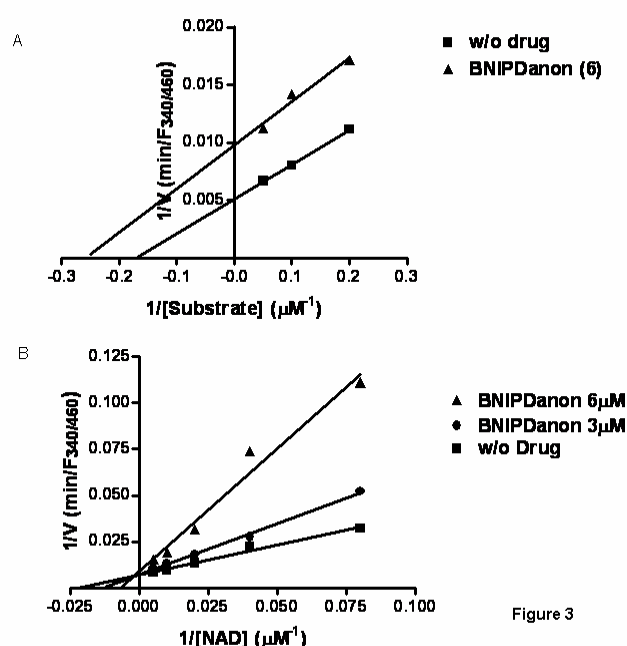


Figure 3

Figure 3. Kinetics of inhibition of LiSIR2RP1 by the BNIPDanon (6). The rate of deacetylation of the substrate in the CycLex SIRT1/Sir2 deacetylase fluorometric kit was measured in the presence or absence of $7.5\mu\text{M}$ BNIPDanon (6). Panel A shows the inhibition by BNIPDanon as a function of NAD^+ concentrations (12.5 , 25 , 50 , 100 , and $200\mu\text{M}$); the concentration of substrate was fixed at $10\mu\text{M}$. Panel B shows the inhibition by BNIPDanon as a function of substrate concentration; the concentration of NAD^+ was fixed at $200\mu\text{M}$.

Examination of the inhibitor binding site

To determine the structural differences between LiSIR2RP1 and hSIRT1, a homology model of both proteins was built and analysed. The homology model of LiSIR2RP1 showed 40% identity with the template human SIRT2 (1j8f). The region from 242-525 amino acids showing 41% identity with the template was modelled out of a total of 747 amino acids in hSIRT1. The evaluation of these models using Procheck revealed that 93% of the residues are in the most favoured regions of Ramachandran Plot for both models, with 0.4% of the residues in disallowed region for hSIRT1. No residue was in disallowed region of Ramachandran Plot for LiSIR2RP1. The overall quality factor of the models was 78% for hSIRT1 and 66% for LiSIR2RP1. Based on this information, the homology models were found to be reliable.

BNIP derivatives were docked into these models to analyze the inhibitory effect of the compounds. It was found that all of these compounds are acting on the NAD^+ binding pocket, hence showing competition with NAD^+ (data not shown), which is in agreement with our experimental data. Structural comparison of the binding pocket of LiSIR2RP1 and hSIRT1 revealed a highly conserved NAD^+ binding site; however, the binding mode of the BNIP derivatives differs considerably between the two proteins (Figure 4). BNIPDanon was found to be the best suited as an inhibitor among all other BNIP derivatives, on account of its lowest docking score and higher interaction with the target. The docking scores of BNIPDanon on LiSIR2RP1 and hSIRT1 are -17.2 and -15.8, and the numbers of hydrogen bond formed are six and five, respectively. In hSIRT1, BNIPDanon interacts with Arg274, Ser442, and Asn465 in A pocket and Gln345 in B pocket and diverges away from C pocket (as shown in Figure 4). BNIPDanon shows interaction with the adenine sub-pocket (A pocket) and C pocket (NAD^+ hydrolysis region) in LiSIR2RP1. The residues involved in hydrogen bonding with BNIPDanon in LiSIR2RP1 include Ala40, Gly216, Asn241 present in the A pocket and Asn125 in C pocket. The C pocket is constituted by residues Ser43, His106, Asp127 and Asn125 and is reported to be involved in polarization and hydrolysis of NAD glycosidic bond, leading to the formation of enzyme-ADP-ribose intermediate, as cited for human SIRT2 (Finnin et al, 2001). The interaction of BNIPDanon with C pocket residues may interfere with NAD^+ hydrolysis, and therefore accounts for its action as an inhibitor. Furthermore, based on a detailed computational analysis, subtle differences in the catalytic and ligand binding domains in the LmSIR2 and the hSIR2 were recently reported, opening the possibility of selectively targeting the parasite enzyme (Kadam et al., 2006).

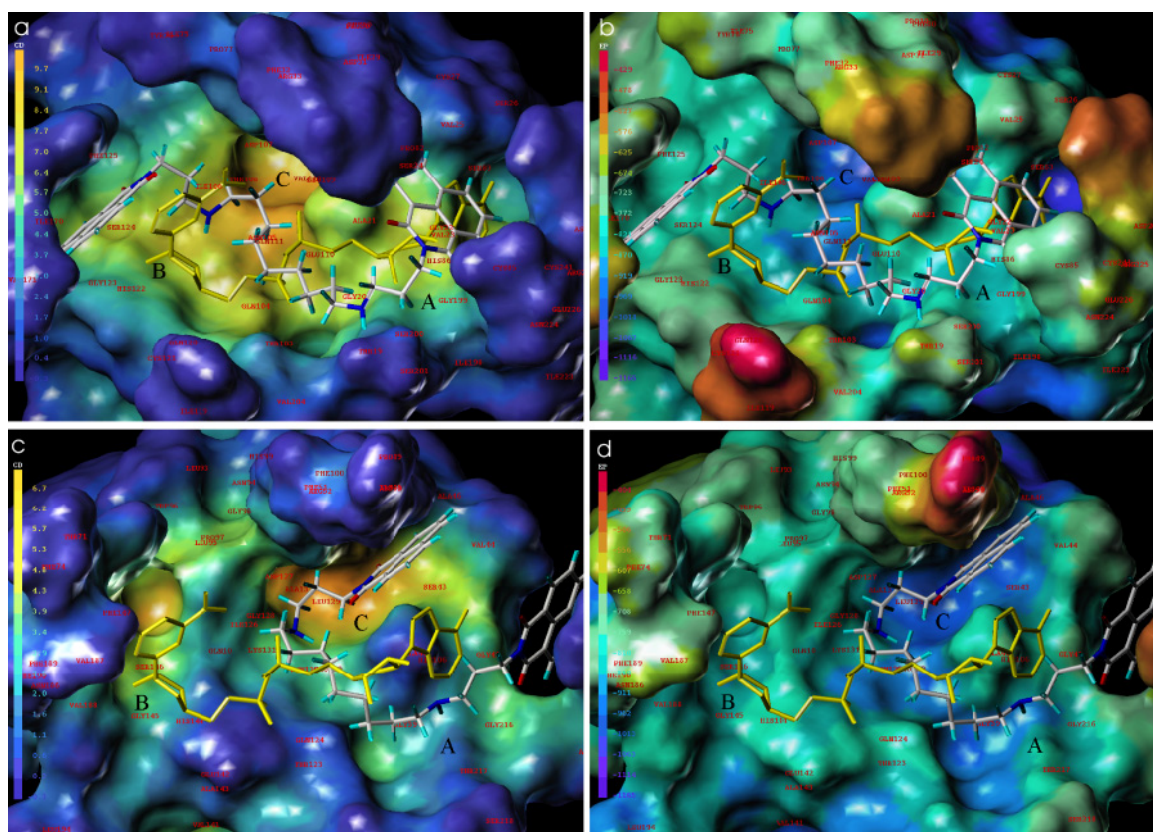


Figure 4- Cavity depth and electrostatic surface analysis for binding pocket of hSIRT1 (A, B) and LiSIR2RP1 (C, D). NAD molecule is colored yellow and BNIPDanon is shown in sticks. The keywords A,B,C in black denote A,B,C pocket of NAD binding site. The homology model of hSIRT1 has 284 amino acids, but the actual protein is 751 amino acid long. Therefore, the residues Arg33, Gln104, Ser201 and Asn224 correspond to Arg274, Gln345, Ser442 and Asn465 in the real sequence.

Conclusion

In this study, BNIP derivatives were for the first time identified as a new class of NAD⁺ competitive SIR2 inhibitors that preferentially inhibit the parasite LiSIR2RP1 enzyme. Besides the identification of a new class of SIR2 inhibitors, this study sustains that among the SIR2 enzymes' catalytic core domain conservation, subtle differences may permit selective targeting. However, concerns about enzyme selectivity and inhibitory potency, which might be influenced by the cellular NAD⁺ concentration, remain the key factors for the development of NAD⁺ competitive SIR2 inhibitors.

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2.8 Anti-leishmanial activity of the bisnaphthalimidopropyl derivatives

Leishmaniasis is a parasitic disease caused by the protozoan parasites of the genus *Leishmania*. Depending on the parasite specie the clinical manifestations vary from localized ulcerative skin lesions to disseminated visceral infection. The latter is the most severe form of the disease, which is fatal when left untreated. However the recommended therapy is far from satisfactory due to the emergence of resistances, severe side effects and the limited efficacy owing to disease exacerbation, mainly associated with compromised immune capability (e.g. HIV co-infection). Bisnaphthalimidopropyl (BNIP) derivatives were recently identified as inhibitors of the *Leishmania* SIR2 related protein 1, which is involved in parasite survival and virulence. In this report we have determined the *in vitro* and *in vivo* antileishmanial activity of several BNIP derivatives. The most active BNIP derivatives, *in vitro*, against *L. infantum* intracellular amastigotes expressing stable luciferase activity, exhibited an IC_{50} ranging from $1.01 \pm 0.39 \mu M$ and $2.43 \pm 0.19 \mu M$. Their capacity to treat VL was evaluated using Balb/C mice chronically infected with *L. infantum* as a model. These experiments led to the identification of BNIPdiaminooctane (BNIPDaoct) as an effective compound to treat VL due to the significant reduction of the spleen and liver parasite loads. Indeed BNIPDaoct was more effective into treat VL upon a short course treatment (3 or 6 drug administrations) than amphotericin B. Moreover, no signals of haematological toxicity were detected.

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Anti-leishmanial activity of the bisenaphthalimidopropyl derivatives

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Abstract

Leishmaniasis is a parasitic disease caused by the protozoan parasites of the genus *Leishmania*. Depending on the parasite specie the clinical manifestations vary from localized ulcerative skin lesions to disseminated visceral infection. The latter is the most severe form of the disease, which is fatal when left untreated. However the recommended therapy is far from satisfactory due to the emergence of resistances, severe side effects and the limited efficacy owing to disease exacerbation, mainly associated with compromised immune capability (e.g. HIV co-infection). Bisenaphthalimidopropyl (BNIP) derivatives were recently identified as inhibitors of the *Leishmania* SIR2 related protein 1, which is involved in parasite survival and virulence. In this report we have determined the in vitro and in vivo antileishmanial activity of several BNIP derivatives. The most active BNIP derivatives, in vitro, against *L. infantum* intracellular amastigotes expressing stable luciferase activity, exhibited an IC₅₀ ranging from 1.01±0.39µM and 2.43±0.19µM. Their capacity to treat VL was evaluated using Balb/C mice chronically infected with *L. infantum* as a model. These experiments led to the identification of BNIPdiaminooctane (BNIPDaoct) as an effective compound to treat VL due to the significant reduction of the spleen and liver parasite loads. Indeed BNIPDaoct was more effective into treat VL upon a short course treatment (3 or 6 drug administrations) than amphotericin B. Moreover, no signals of haematological toxicity were detected.

Introduction

Leishmaniasis is a parasitic disease caused by the protozoan parasites of the genus *Leishmania*. Depending on the parasite species the clinical manifestations vary from localized ulcerative skin lesions to disseminated visceral infection. The latter is the most severe form of the disease, which is fatal when left untreated. A vertebrate host becomes infected with *Leishmania*, through the bite of an infected sandfly (*Phlebotomus* and *Lutzomyia spp*) along with its blood meal, by the inoculation of infective flagellated promastigotes that invade or are phagocytosed by local or recruited host cells. In the phagolysosomes, the promastigotes will differentiate into non-flagellated amastigotes that multiply and are able to infect other adjacent or distant macrophages.

The disease control is dependent on drug therapy once no approved human vaccine is available. Pentavalent antimonials, represented by sodium stibogluconate and meglumine antimoniate, have been the first-line treatment in many endemic areas for more than 7 decades (Chappuis et al., 2007). Among the antimonials severe adverse effects, are the life-threatening acute pancreatitis and cardiac arrhythmia. Amphotericin B has replaced antimonials as first-line treatment in many areas of the Bihar State in India, where treatment unresponsiveness is more than 60% (Sundar et al., 2000). Although, its administration requires close medical supervision due to high toxicity (fever with rigor and chills, thrombophlebitis and occasional serious toxicities like myocarditis, severe hypokalaemia, renal dysfunction and even death) (Sundar et al., 2006). The toxic events were substantially reduced by the emergence of Amphotericin B lipid formulations (Sundar et al., 2000; Sundar et al., 2004). Indeed, these formulations allowed a targeted drug delivery to parasites, once they are preferentially internalised by the reticuloendothelial cells. Moreover liposomal formulations of amphotericin B have been considered as the best treatment option to VL. Even these formulations are used as first-line treatment in Europe and United States, its use in endemic areas was precluded due to economic reasons (Gradoni et al., 2003; Bern et al., 2006). Recently introduced in India, miltefosine is the first oral drug to treat VL. It is highly effective (adults and children's) and well tolerated, with limited side effects that are usually mild and temporary (Sundar et al., 1998; Sundar et al., 1999; Jha et al., 1999; Sundar et al., 2000; Sundar et al., 2002; Bhattacharya et al., 2004). However, miltefosine is teratogenic, being its use strictly forbidden in pregnant women's or in women's who could become pregnant within two months of treatment (Sundermann et al., 2006). Indeed, the recommended therapy is far from satisfactory, therefore the search for new drugs or formulations against VL is

sustained by several reasons including the emergence of resistances, severe side effects and the limited efficacy owing to disease exacerbation, mainly associated with compromised immune capability (e.g. HIV co-infection) (Croft et al., 2006). Indeed, greater attention should be paid to this last point, since HIV-infected patients usually develop asymptomatic visceral infections, widespread atypical organ infections, reduced responsiveness to chemotherapy and high relapse rates when the treatment is discontinued (Pintado et al., 2001; Lyons et al., 2003; Morales et al., 2002).

The proteins belonging to the Silent Information Regulator 2 (SIR2) family also known as sirtuins, have been involved in the regulation of a number of biological processes such as heterochromatin formation, gene silencing, DNA repair, development, longevity, metabolism, adipogenesis, and apoptosis (reviewed in Zhao and Marmorstein, 2006). During the last few years, we have been interested in the protozoan parasite *Leishmania infantum* SIR2 related protein 1, LiSIR2RP1, due to its role in parasite's survival and virulence (Vergnes et al., 2002; 2005). Indeed, this cytosolic parasite protein, partially associated with the microtubule network, is a tubulin NAD⁺ dependent deacetylase and ADP-ribosyltransferase (Tavares et al., 2008). The search for target specific LiSIR2RP1 inhibitors led to the identification of bisnaphthalimidopropyl derivatives as a new class of sirtuins inhibitors (Tavares et al., submitted). Furthermore, in a previous study we have shown that bisnaphthalimidopropyl (BNIP) polyamine derivatives exerted in vitro anti-*Leishmania* activity, inducing the death of promastigote forms by apoptosis (Tavares et al., 2005).

In this report we have determined the in vitro anti-leishmanial activity of several BNIP derivatives against *L. infantum* promastigotes, axenic and intracellular amastigotes, expressing stable luciferase activity. The most active compounds against intracellular amastigotes were selected for in vivo experiments, and their capacity to treat VL was evaluated using Balb/C mice infected with *L. infantum* as a model. Indeed, we demonstrated for the first time that, treatment of infected mice with intraperitoneal injections of BNIPdiaminooctane (BNIPDaooct) significantly reduced the parasite load in the liver and spleen, compared with the parasite load of animals that received only the drug vehicle. Moreover, no signals of haematological toxicity were detected.

Material and Methods

Compounds

The polyamine derivative compounds assigned as bisnaphthalimidopropyl putrescine (BNIPPut), spermidine (BNIPSpd) and spermine (BNIPSpm) were synthesized as described previously (Lin and Pavlov, 2000). The bisnaphthalimidopropyldiamino (BNIPDa) derivatives such as octane (BNIPDaoct), nonane (BNIPDanon), decane (BNIPDadec) and the bisnaphthalimidopropyldipropyltriamine (BNIPDpta) and bisnaphthalimidopropyldiethyltriamine (BNIPDeta) were synthesized as described previously (Oliveira J. et al., 2007). The BNIPDa derivatives such as pentane (BNIPDapen), hexane (BNIPDahex), heptane (BNIPDahep) and dodecane (BNIPDadodec) were synthesized as described previously (Tavares et al., 2008, submitted). Amphotericin B was purchased from Sigma. All of the compounds stock solutions were prepared in DMSO and stored at -20°C.

Parasites

A cloned line of *L. infantum* (MHOM/MA/67/ITMAP-263) wild type (wt) promastigotes was grown at 27°C in RPMI 1640 medium (Cambrex) supplemented with 10% heat inactivated foetal bovine serum (FBS-Cambrex), 2mM L-glutamine (Cambrex), 20mM Hepes (Cambrex), 100U/ml penicillin (Cambrex) and 100µg/ml streptomycin (Cambrex). Axenic amastigote forms were grown at 37°C with 5% CO₂ in a cell free medium called MAA/20 (medium for axenic amastigotes). MAA/20 consisted of modified medium 199 (GIBCO) with Hank's balanced salt solution supplemented with 0.5% trypto-casein (Oxoid), 15mM D-glucose (SIGMA), 5mM glutamine (Cambrex), 4mM NaHCO₃ (Sigma), 0.023mM bovine hemin (FLUKA), and 25mM HEPES to a pH of 5.8 and supplemented with 20% heat inactivated FBS (Cambrex). A cloned line of *L. infantum* expressing the luciferase gene (LUC) and derived from wt was used in all the in vitro experiments (Serenio et al., 2001).

Growth inhibition assays

The *L. infantum* expressing-LUC promastigotes and axenic amastigotes, (10⁶/ml), were incubated with a serial range of concentrations of each drug for 3 days at 27°C or 37°C respectively. The growth of the LUC-expressing amastigotes in the human leukaemia monocyte cell line (THP-1 cells) was evaluated according to Serenio et al., 2001 with some modifications. Briefly, THP-1 cells were cultured in RPMI 1640 medium (Cambrex) supplemented with 10% heat inactivated foetal bovine serum (FBS-Cambrex), 2mM L-glutamine (Cambrex), 100U/ml penicillin

(Cambrex) and 100 μ g/ml streptomycin (Cambrex). Log phase THP-1 cells, were differentiated by incubation for 2 days in medium containing 20ng/ml of PMA (Sigma). Once differentiated, the cells became adherent and were washed with prewarmed medium and then infected with stationary expressing-LUC axenic amastigotes at a parasite/macrophage ratio of 3:1 during 4 hours at 37°C with 5% CO₂. Non internalized parasites were removed and serial dilutions of each drug were added. After 3 days of incubation at 37°C, 5% CO₂ the cells were washed and the luciferase activity was determined.

The luciferase activity of the LUC-expressing parasites was determined as described elsewhere (Roy et al., 2000) and the values were expressed as relative light units (RLU). The percentage of growth inhibition was calculated as (1-RLU of drug treated parasites/RLU drug untreated parasites) x100. The IC₅₀ (concentration required to inhibit the growth by 50%) was determined by linear regression analysis.

Mice infection and drug treatments

Five-week-old BALB/c male mice were obtained from Charles River (Spain) and maintained at the animal facilities of the Instituto de Biologia Molecular e Celular (IBMC, Porto, Portugal). Mice were kept five per cage and allowed food and water ad libitum. The mice at seven weeks of age were infected with 10⁸ wt *L. infantum* promastigotes. For that, a stationary phase promastigotes culture, were collected, washed, and resuspended in 200 μ l of sterile PBS and then i.p. injected on mice. After 8 weeks post-infection, the animals (n=3 or n=4) received 1mg/kg of each drug diluted in 200 μ l sterile PBS by i.p. injection during 3, 6, 9, 12 or 15 days consecutively. The control group received PBS containing the same amount of DMSO (0.31%, v/v) that is present in the preparation of each drug tested. 3 days after the last drug administration the animals were euthanized and the parasite load evaluated in the liver and spleen as described below.

Parasite load quantification

The parasite load in the liver and spleen was determined by the limiting dilution method as previously described (Buffet et al., 1995). Briefly the organs were removed, homogenized and resuspended in RPMI supplemented with 10% heat inactivated FBS, 2mM L-glutamine, 20mM Hepes, 100U/ml penicillin and 100 μ g/ml streptomycin. The organs were then submitted in quadruplicate, to the serial 2-fold dilutions in a 96 well microtitration plates. The plates were incubated at 27°C for 15 days and then each well was inspected for the presence or absence of promastigotes. The final titter was the last dilution for which, at least one

promastigote was recorded. The number of parasites per gram of organ (parasite load) was calculated as follows: parasite load= [(geometric mean of reciprocal titer from each quadruplicate cell culture/weight of homogenized organ) x reciprocal fraction of the homogenized organ inoculated into the first well].

Blood analysis

Uncoagulated blood samples were used to obtain a complete blood evaluation, including total red blood cell, haemoglobin, hematocrit, total white blood cells, and granulocyte, monocyte, lymphocyte counts.

Statistical analysis

The data were analyzed using Student's t-test.

Results and discussion

In vitro effect of BNIP derivatives on L. infantum promastigotes, axenic and intracellular amastigotes expressing stable luciferase activity.

The several *L. infantum* parasite forms, including promastigotes, axenic and intracellular amastigotes, were treated with 12 BNIP derivative compounds in order to evaluate its antileishmanial activity. Basically, the several BNIP derivatives differed in the alkyl length of the central chain containing 2, 3 or 4N atoms, linking the 2 naphthalimidopropyl groups (Lin and Pavlov, 2000; Oliveira J et al., 2007; Tavares J et al., submitted). The inhibition of the parasites growth induced by each drug were evaluated by measuring the activity of the reporter protein, luciferase. Indeed, this assay was previously validated to be used in drug screening experiments (Roy et al., 2000; Sereno et al., 2001). The use of parasites transfected with reporter genes, such as luciferase in drug screening assays, represents a meaningful toll to determine drug efficacy in intracellular amastigotes once these determinations were classically performed by direct counting, which is very laborious, time consuming and may give inaccurate determination of the IC₅₀ through the difficulty to evaluate parasite viability by a staining procedure.

All compounds were tested in non-infected human monocyte derived macrophages, before being tested against intracellular amastigotes, and the cytotoxicity evaluated by the MTT assay. Indeed, all of the drugs concentrations that were toxic against non-infected macrophages were not used in the assays to determine the IC₅₀ against intracellular amastigotes (data not shown). Generally, the several BNIP

derivative compounds were more active, as evaluated by the IC_{50} values, against promastigotes, followed by intracellular amastigotes and less active against axenic amastigotes (Table I). The most active compound against the intracellular amastigotes was the BNIPDadodec (IC_{50} $1.01 \pm 0.39 \mu M$) while the less active compound was the BNIPDeta (IC_{50} $9.52 \pm 0.56 \mu M$). Even, the compounds potency was lower against axenic amastigotes compared to intracellular amastigotes, the more active compound remained the BNIPDadodec (IC_{50} $4.03 \pm 0.69 \mu M$). Moreover, the drugs activity against axenic and intracellular amastigotes, were dramatically different in the case of the BNIPDapen, BNIPDahehex, BNIPDahep and the BNIPDaoct, which IC_{50} values against axenic and intracellular amastigotes were respectively: $22.61 \pm 1.99 \mu M$ and $1.26 \pm 0.18 \mu M$ (BNIPDapen); $>50 \mu M$ and $3.46 \pm 0.48 \mu M$ (BNIPDahehex); $29.80 \pm 1.61 \mu M$ and $1.12 \pm 0.0084 \mu M$ (BNIPDahep); $>50 \mu M$ and $2.43 \pm 0.19 \mu M$ (BNIPDaoct). The possibility to culture some species of *Leishmania* amastigotes, under axenic conditions, represented a major advance in drug screenings, which were usually performed with the parasite insect stage, the promastigotes (Bates, 1994; Balanco et al., 1998; Sereno et al., 1997; Ephros et al., 1999). Meanwhile differences in drug sensitivity between axenic and intracellular amastigotes were already reported, supporting the necessity of evaluate drugs efficacy against the intracellular forms (Ephros et al., 1999). Indeed, some explanations could be attributed to such differences, including an antileishmanial effect mediated by the host cell or alternatively, an interference with the high concentration of serum used in the axenic amastigotes culture medium.

The more active compounds against the intracellular amastigotes, assigned as BNIPDapen, BNIPDahep, BNIPDaoct, BNIPDadec, BNIPDadodec, were selected for the in vivo experiments.

Table 1. IC₅₀±SD (μM) of BNIP derivatives in *L. infantum* expressing-LUC promastigotes, axenic amastigotes and intracellular amastigotes.

Compound	Promastigotes	Axenic Amastigotes	Intracellular Amastigotes
BNIPDabut	0.40±0.15	5.49±0.67	4.53±0.54
BNIPDapen	0.52±0.071	22.61±1.99	1.26±0.18
BNIPDahep	0.52±0.010	>50	3.46±0.48
BNIPDahep	0.98±0.21	29.80±1.61	1.12±0.0084
BNIPDaoct	0.19±0.11	>50	2.43±0.19
BNIPDanon	2.09±0.54	17.42±0.97	6.03±0.67
BNIPDadec	0.46±0.016	4.47±0.19	1.02±0.41
BNIPDadodec	0.40±0.15	4.03±0.69	1.01±0.39
BNIPDpta	1.09±0.12	5.24±0.93	4.22±1.07
BNIPDeta	0.96±0.17	6.97±0.20	9.52±0.56

Promastigotes, axenic amastigotes or PMA differentiated THP-1 cells infected with amastigotes were incubated with a serial range of each drug concentrations during three days. The growth inhibitory effect of the drugs was determined by the luciferase assay and the IC₅₀ calculated by linear regression analysis. Each experiment was performed in triplicate and the IC₅₀ values represented correspond to the mean value obtained for at least three independent experiments.

In vivo antileishmanial activity of BNIP derivatives

The antileishmanial activity of BNIP derivatives (1mg/kg) (BNIPDapen, BNIPDahep, BNIPDaoct, BNIPDadec and BNIPDadodec) was investigated in vivo against VL by the reduction of the spleen and liver parasite load of Balb/C mice chronically infected with *L. infantum*. A group of mice receiving 1mg/kg of the antileishmanial drug amphotericin B and another group receiving only the drug vehicle (PBS containing 0.31% of DMSO) were included as a controls. All the animals were treated during 3 consecutive days and the drug efficacy was evaluated 3 days after the last drug administration. Among the several treatments, the administration of BNIPDahep and BNIPDaoct, led to a significative (p<0.05) reduction of the spleen parasite load compared to the group that received only the drug vehicle (Figure 1). Indeed, the parasite load ± SD [Log (parasites.g⁻¹)] was reduced from 6.11±0.35 (mice that received the drug vehicle) to 5.11±0.30 and 5.10±0.30 in the case of mice treated respectively with BNIPDahep and BNIPDaoct. However, only the treatment with the BNIPDaoct led to a significative (p<0.001) reduction of the liver parasite load ± SD [Log (parasites.g⁻¹)] that decreased from 4.41±0.17 (mice that

received the drug vehicle) to 3.11 ± 0.16 (mice treated with BNIPDaoct). The administration of amphotericin B also slightly reduced the liver parasite load to 3.80 ± 0.35 [Log (parasites.g⁻¹)]. A different scheme of treatment through the administration of the several drugs at 1mg/kg twice a day and during the same period of time as the above treatments (3 consecutive days), was also conducted. However no increased reduction of the parasite load was detected (data not shown).

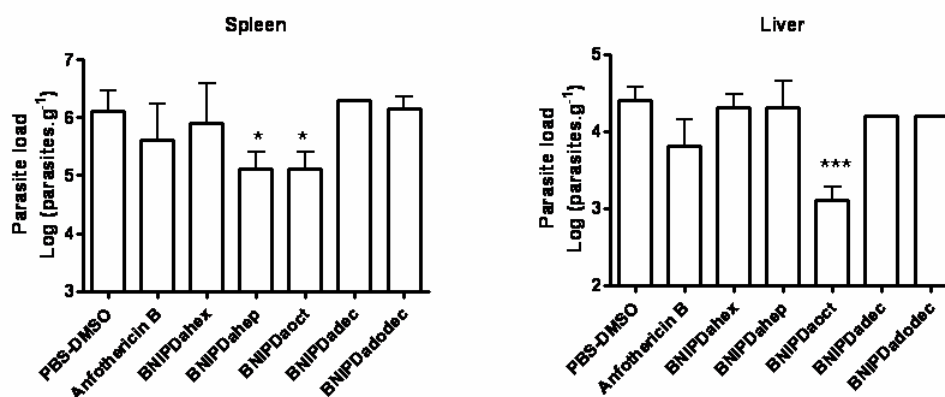


Figure 1. Comparative analysis of the parasite load in the spleen (A) and liver (B) of BALB/c mice infected with *L. infantum* and treated with different drugs. The mice, 8 weeks post-infection with 10^8 promastigotes, were treated i.p. with 1mg/kg of the following drugs: Amphotericin B, BNIPDapen, BNIPDahep, BNIPDaoct, BNIPDadec and BNIPDadodec during 3 consecutive days. The control group received PBS containing 0.31% (v/v) DMSO. The drugs efficacy was evaluated 3 days after the last treatment. The results are representative of three experiments conducted independently. *, $p < 0.05$; ***, $p < 0.001$ between the drug treated group and the control group.

Blood samples were collected 3 days after the last treatment for haematological toxicity analysis. Uncoagulated blood samples were used to obtain a complete blood evaluation, including red blood cells, total white blood cells, haemoglobin, hematocrit, and lymphocyte, neutrophil and monocyte count. Table II shows the effect of intraperitoneal administration of 1mg/kg/day during three consecutive days of BNIPDapen, BNIPDahep, BNIPDaoct, BNIPDadec and BNIPDadodec to *L. infantum* infected Balb/C mice. Moreover the table II also includes data from the treatment with the antileishmanial drug amphotericin B and the drug vehicle (PBS containing 0.31% of DMSO). Treatment with BNIPDadodec caused normochromic anemia, as evidenced by the significant ($p < 0.01$ or $p < 0.05$) decrease in the proportions of total red blood cells [6.35 ± 0.88 to 9.55 ± 0.74 ($10^6/\text{mm}^3$)], haemoglobin [10.5 ± 1.2 to 14.8 ± 0.9 (g/dl)] and hematocrit (32.9 ± 3.3 to 48.6 ± 4.5) compared with the values of the group receiving the drug vehicle.

Table II. Hematologic changes after 3 days of consecutive administrations of 1mg/kg of each drug to *L. infantum* infected BALB/c mice.

Treatment	White blood cell count (10³/mm³)	Red blood cell count (10⁶/mm³)	Hemoglobin concentration (g/dl)	Hematocrit	Lymphocyte count (10³/mm³)	Monocyte count (10³/mm³)	Granulocyte count (10³/mm³)
PBS-DMSO	3.53±0.55	9.55±0.74	14.8±0.9	48.6±4.5	2.30±0.30	0.63±0.11	0.60±0.17
Amphotericin B	4.5±0.28	8.25±1.11	13.4±1.7	41.3±5.7	3.15±0.64	1.10±0.28**	0.25±0.071
BNIPDa _{hex}	4.95±0.21*	8.81±0.66	13.9±0.7	44.2±3.8	3.25±0.21	1.15±0.21	0.55±0.21
BNIPDa _{hep}	3.15±0.78	9.93±0.08	15.6±0.1	50.5±0.7	2.35±0.49	0.25±0.07**	0.55±0.21
BNIPDa _{oct}	5.30±0.99*	9.33±0.078	14.8±0.7	47.4±1.3	3.83±0.93	0.90±0.10	0.60±0.10
BNIPDa _{dec}	2.87±0.40	7.12±1.76	11.6±2.6	39.4±7.1	1.87±0.67	0.85±0.30	0.30±0.1
BNIPDa _{dodec}	2.43±0.76	6.35±0.88**	10.5±1.2**	32.9±3.3*	1.67±0.59*	0.63±0.25	0.13±0.058*

The values represent the mean ± standard deviation of the means of 3 to 4 mice per group and are representative of three experiments conducted independently. *, p<0.05; ***, p<0.001 between the drug treated group and the control group.

This treatment also induced a significant ($p < 0.05$) reduction in the lymphocyte [1.67 ± 0.59 to 2.30 ± 0.30 ($10^3/\text{mm}^3$)] and granulocyte [0.13 ± 0.058 to 0.60 ± 0.17 ($10^3/\text{mm}^3$)] counts compared to the control group. Moreover a similar effect, even not significant, was obtained by treatment of mice with BNIPDadec. Indeed, these alterations in the blood parameters suggest that the drugs (BNIPDadec and BNIPDadodec) may accumulate in the blood therefore have different tissue distribution compared to BNIPDahep or BNIPDaoct. These could also be an explanation to their lack of in vivo antileishmanial effect as observed by the absence of effect in reducing the spleen and liver parasite loads.

An increase in the white blood cells counts ($10^3/\text{mm}^3$) was detected in the mice groups treated with BNIPDahex (4.95 ± 0.21), BNIPDaoct (5.30 ± 0.99) and Amphotericin B (4.50 ± 0.28), even the increased of the later was not significant, compared to the control group (3.53 ± 0.55).

The promising in vivo antileishmanial activity exhibited by the BNIPDaoct derivative led us to evaluate whether by increasing the number of drug administrations it would be possible to improve its efficacy. Three groups of mice chronically infected with *L. infantum*, received three different treatments (drug vehicle; 1mg/kg/day of BNIPDaoct or Amphotericin B) during 3, 6, 9, 12 or 15 days consecutive days. Similarly to the previous experiments the treatment efficacy was evaluated through the decrease of the parasite load evaluated three days after the last drug administration.

The efficacy of the BNIPDaoct to reduce the spleen parasite load was comparable to amphotericin B upon 9, 12 or 15 days of treatment (Figure 2). However, BNIPDaoct was more effective than amphotericin B in reducing the spleen parasite load under a short period of treatment (3 or 6 administrations).

Concerning the liver parasite loads, 3 administrations of BNIPDaoct were sufficient to achieve a significant reduction ($p < 0.001$) compared to the control group (Figure 2). Indeed, subsequent administrations of this drug didn't increase the reduction of the liver parasite load. The reduction of the liver parasite load by amphotericin B increased with the number of administrations. Indeed, 9 administrations of amphotericin B were required to reduce the liver parasite load to the same level that was achieved with 3 administrations of BNIPDaoct.

Interestingly, 6 administrations of both drugs, BNIPDaoct and Amphotericin B, led to a significant increase in the white blood cells counts ($10^3/\text{mm}^3$) on peripheral blood to 5.86 ± 0.03 and 7.40 ± 0.01 respectively, compared to 4.37 ± 0.76 of the control group (Table III).

Table III. Hematologic changes after 6, 9, 12 or 15 days of consecutive administrations of 1mg/kg of each drug to *L. infantum* infected BALB/c mice.

Number Administrations	Treatment	White blood cell count (10³/mm³)	Red blood cell count (10⁶/mm³)	Hemoglobin concentration (g/dl)	Hematocrit	Lymphocyte count (10³/mm³)	Monocyte count (10³/mm³)	Granulocyte count (10³/mm³)
6	PBS-DMSO	4.37±0.76	8.60±0.45	14.2±0.7	42.4±1.6	3.23±0.57	0.70±0.20	0.43±0.15
	Amphotericin B	7.40±0.01*	9.10±0.032	14.5±0.7	45.6±0.2*	5.35±0.07*	1.30±0.0*	0.75±0.07
	BNIPDaact	5.8±0.03*	8.32±0.35	13.8±0.5	41.3±2.1	4.30±0.26*	1.03±0.15	0.47±0.15
9	PBS-DMSO	3.20±0.57	8.42±0.70	13.7±1.3	41.9±4.7	2.25±0.49	0.50±0.14	0.45±0.070
	Amphotericin B	4.10±0.28	8.96±0.28	14.1±0.0	45.0±1.8	2.90±0.56	0.65±0.21	0.55±0.07
	BNIPDaact	2.57±0.38	7.36±0.98	11.6±1.7	36.3±5.3	2.00±0.20	0.30±0.17	0.27±0.058
12	DMSO	4.38±1.30	9.46±0.42	14.5±0.7	46.9±2.2	3.33±0.49	0.60±0.18	0.45±0.060
	Amphotericin B	3.33±0.29	9.11 ±0.69	14.7±0.8	45.5±3.4	2.37±0.15	0.43±0.06	0.53±0.15
	BNIPDaact	2.40±0.84	8.50±0.23	13.6±0.2	42.1±1.6*	1.55±0.49	0.50±0.28	0.35±0.071
15	DMSO	3.60±0.28	8.87±0.17	14.3±0.2	45.0±0.1	2.25±0.21	0.90±0.06	0.5±0.0
	Amphotericin B	6.03±1.09	9.25±0.26	15.0±0.3	48.0±1.5	4.17±0.60*	1.30±0.38	0.77±0.25
	BNIPDaact	2.10±0.78	7.92±1.37	12.6±1.4	40.7±7.7	1.37±0.31*	0.40±0.30	0.33±0.23

Values represent the mean ± standard deviation of the means of 3 to 4 mice per group. *, p<0.05; ***, p<0.001 between the drug treated group and the control group.

Such increase was due to a raise in the numbers of lymphocytes and monocytes. No signals of haematological toxicity were detected in the different treatments even upon several administrations. However, the continued administration of BNIPDaoct led to a slight reduction in the number of white blood cells compared to the control group. Indeed, following 15 administrations of BNIPDaoct a significant ($p < 0.05$) reduction in the lymphocyte counts, 1.37 ± 0.31 ($10^3/\text{mm}^3$), were detected compared to the control group 2.25 ± 0.21 ($10^3/\text{mm}^3$) (Table III).

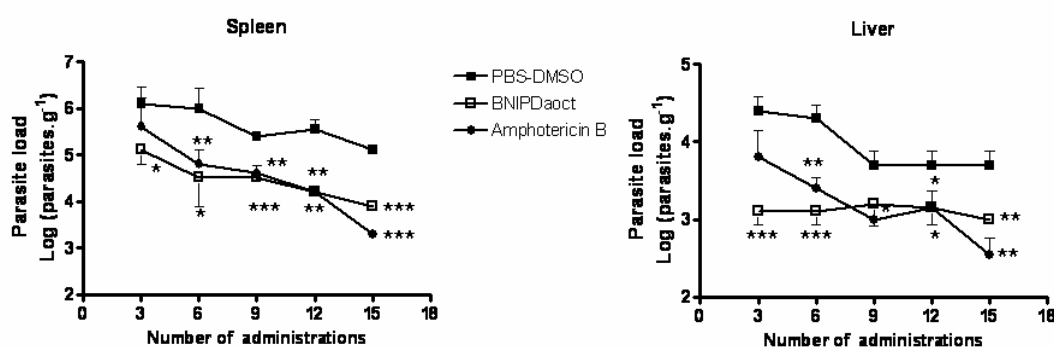


Figure 2. Progression of the parasite load in the spleen (A) and liver (B) during drug treatment. The mice, 8 weeks post-infection with 10^8 promastigotes, were treated daily by i.p. injection with 1mg/kg Amphotericin B or 1mg/kg BNIPDaoct during 3, 6, 9, 12 or 15 consecutive days. The control group received PBS containing 0.31% (v/v) DMSO. The drugs efficacy was evaluated 3 days after the last treatment of 3, 6, 9, 12 or 15. The results are representative of three experiments conducted independently. *, $p < 0.05$; ***, $p < 0.001$ between the drug treated group and the control group.

Concluding Remarks

On this report we have demonstrated the potential value of BNIP derivatives as antileishmanial drugs and identified the BNIPDaoct derivative as an effective drug to treat VL. The comparative in vitro analysis of the capacity of BNIP derivatives to inhibit the growth of the different parasite forms (promastigotes, axenic and intracellular amastigotes) sustained the necessity to include the intracellular amastigotes in the drug screening assays. Indeed significant differences between axenic and intracellular amastigotes sensitivity to some BNIP derivatives were detected. Even axenic amastigotes were already validated to be used in drug screening assays exhibiting similar drug-sensitivity as intracellular amastigotes (Callahan et al., 1997; Sereno et al., 1997) differences in drug sensitivity between *L. donovani* axenic and intracellular amastigotes were previously reported (Ephros et al., 1999).

The administration, of the most active in vitro BNIP derivatives, in a mice model of VL enabled the determination of the drug activity in relation to absorption, distribution, metabolism, excretion and gave an early indication about potential toxicity. Indeed, the BNIPDaoct exerted significant antileishmanial activity in vivo even through a short course treatment (3 or 6 administrations) without inducing haematological toxicity. However, the treatment did not lead to completely cure of *Leishmania* infection. Among the several explanations could be the drug difficulty into gain access to all organ infected areas. Further studies containing the drug inside of target delivery systems, such as liposomes, will be useful.

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Chapter VI

Discussion and perspectives

1 *Leishmania* SIR2 homologue functions: proven and predicted

The advances in molecular biology have made available novel approaches in the identification of parasite virulence factors, such as forward and reverse genetics. A genetic approach through the disruption of the *Leishmania* SIR2 encoding gene was conducted to determine the biological role of this protein in the parasite. The impossibility of generating null chromosomal mutants (LiSIR2^{-/-}) without requiring episomal rescue, suggested that the cytosolic SIR2 homologue *Leishmania* protein was determinant to parasite survival. Furthermore, single LiSIR2 gene disruption (LiSIR2^{+/-}) did not affect the growth of parasites in the promastigote form, while amastigotes displayed a marked reduction in their capacity to multiply *in vitro*, both axenically and inside macrophages, and *in vivo* in Balb/c mice. These findings led us to consider the LiSIR2 protein as an attractive and potential therapeutic target. Indeed, the ability to modify the *Leishmania* genome by introducing or eliminating genes has also been considered a powerful strategy to develop a new generation of vaccines against leishmaniasis. The attenuated virulence exhibited by the LiSIR2^{+/-} single mutant parasite in a vertebrate host led to the testing of its ability to provide protection against a virulent *L. infantum* challenge. Accordingly, vaccination with a single intraperitoneal injection of LiSIR2^{+/-} mutant parasites elicited complete protection. Reversal of T cell anergy with specific anti-*Leishmania* cytotoxic activity, high levels of NO production, production of anti-*Leishmania* specific antibodies, and an increased *Leishmania*-specific INF γ /IL-10 ratio, were shown in vaccinated animals (Silvestre et al., 2007).

Despite SIR2 proteins' conservation throughout evolution, the only one of them that has proved essential to growth, so far, is the *L. infantum* cytosolic SIR2 related protein 1. Indeed, in the case of the protozoa parasite *T. brucei*, SIR2rp1 null mutants were generated displaying neither growth nor differentiation (blood stage to insect vector stage) defects (Alsford et al., 2007). Moreover, these *T. brucei* and *Leishmania* proteins appear to be homologues (59% identity), which contrasts with such distinct functions. The disruption of the two other *T. brucei*, SIR2rp2 and SIR2rp3 genes, encoding two mitochondria-localized SIR2 homologues, affected neither growth nor parasite differentiation (Alsford et al., 2007). Furthermore, disruption of the gene encoding a SIR2 homologue in *P. falciparum*, has disclosed its role in the regulation of virulent genes located in the subtelomeric regions, such

as the *var* and *rifin* gene families (Duraisingh et al., 2005). However, like TbSIR2rp1 and PfSIR2, we have demonstrated that LiSIR2RP1 exhibits robust NAD⁺-dependent deacetylase and ADP-ribosyltransferase activities (Garcia-Salcedo et al., 2003; Merrick et al., 2007). All of these parasite enzymes were capable of strongly ADP-ribosylating histones. However, this was only attributed a biological role in the case of TbSIR2rp1. Indeed, it has been suggested that histones' ADP-ribosylation mediated by TbSIR2rp1 is involved in DNA repair due to a correlation between the level of TbSIR2rp1 expression and the resistance to DNA damage (Garcia-Salcedo et al., 2003). These bi-functional enzymatic activities attributed to parasite enzymes contrast with other Sirtuins already characterized. Indeed, it is interesting to speculate whether this is related to the relative paucity of sirtuins in parasites, especially because LiSIR2RP1 and TbSIR2RP1 fall in a phylogenetic class different from that of PfSIR2, and are not closely homologous (Garcia-Salcedo et al., 2003; Merrick et al., 2007). From the seven human sirtuins, only SIRT2 is often localized in the cytoplasm (North et al., 2003; Michishita et al., 2005). Indeed, three human sirtuins (SIRT1, SIRT6 and SIRT7) are localized in the nucleus, presenting distinct subnuclear localizations, since SIRT6 is associated with telomeres and SIRT7 is localized in the nucleoli (Michishita et al., 2005; Michishita et al., 2008). SIRT3, SIRT4 and SIRT5 are localized in mitochondria (Michishita et al., 2005). However, SIRT1's cytosolic localization has been reported and the nucleocytoplasmic shuttling proposed, as a SIRT1 function regulator mechanism (Moynihan et al., 2005; Tanno et al., 2007). Even though mostly residing in cytosol, where it functions as a tubulin NAD⁺-dependent deacetylase, SIRT2 has also been observed in the nucleus (North et al., 2003; Bae et al., 2004). Indeed, a role in the regulation of mitotic exit in cell-cycle has been attributed to this protein (Dryden et al., 2003). This was based in the increased SIRT2 abundance during mitosis and its multiple phosphorylation at the G2/M transition of cell-cycle. Moreover, the phosphatase, CDC14B, may provoke exit from mitosis coincident with the loss of SIRT2 via ubiquitination and subsequent degradation by the 26S proteasome (Dryden et al., 2003). A recent study has revealed that SIRT2 is a substrate of cyclin-dependent kinases (Cdks) which regulate SIRT2 function, since phosphorylation of the Serine-331 inhibits its catalytic activity (Pandithage et al., 2008). SIRT2 maintains a largely cytosolic localization during interphase, due to an active nuclear export by the presence of a Crm-1 dependent nuclear export signal in the protein sequence. Furthermore, during the cell cycle, SIRT2 becomes enriched in the nucleus and is associated with mitotic structures, beginning with the centrosome during prophase, the mitotic spindle during metaphase, and the midbody during cytokinesis (North and Verdin, 2007).

HDAC6 included in the NAD⁺-independent class IIb, is a unique HDAC localized in the cytosol, where it associates with non-histone substrates such as tubulin and HSP90. It has two catalytic domains and an ubiquitin binding domain (Zhang et al., 2006; Zou et al., 2006). The overexpression of HDAC6 leads to the deacetylation of α -tubulin and increases cell motility (Hubbert et al., 2002; Haggarty et al., 2003). HDAC6 can not only bind both mono and poly-ubiquitinated proteins, but also promote its own mono-ubiquitination. Specific inhibition of HDAC6 activity or its downregulation by siRNA increases α -tubulin and HSP90 acetylation, which reduces cellular motility and induces the degradation of HSP90 client proteins, cell growth inhibition and cell death (Bali et al., 2005; Kovacs et al., 2005). Similarly to SIRT2, we have found that LiSIR2RP1 is a α -tubulin NAD⁺-dependent deacetylase. Localized in the cytosol, LiSIR2RP1 is partially associated with the parasite's cytoskeleton. The latter is composed mainly of microtubules that are polymers of repeating α/β tubulin heterodimers, and a variety of minor components known as microtubule-associated proteins. Microtubules play an important role in many cellular processes such as mitosis, cell shape, motility, intracellular vesicle transport and organelle position. Moreover, the protozoan parasite *Leishmania*, having a digenetic life cycle, exhibits a particular range of cell shapes mostly defined by their internal cytoskeleton. LiSIR2RP1 is capable of deacetylating both purified tubulin and tubulin in the promastigote or amastigote total extract. However, the disruption of a single allele in the LiSIR2RP1 gene did not significantly affect the levels of total tubulin acetylation in parasites, as evaluated by the ratio of acetylated tubulin over total α -tubulin in the wild type and single LiSIR2RP1 knockout promastigotes and axenic amastigotes. Only a slight difference in the ratio of acetylated tubulin over total α -tubulin between the wild type and single knockout axenic amastigotes was detected. Furthermore, the concomitant presence of LiSIR2RP1 and highly acetylated α -tubulin suggests that tubulin deacetylation could be a transient phenomenon. A similar observation was made by North and Verdin (2007), who described the presence of SIRT2 in mitotic structures, which are mainly composed of microtubules that unexpectedly revealed to be hyperacetylated. Even though α -tubulin acetylation was already reported a few years ago, its importance in cell biological processes is not yet completely understood. However, the level of tubulin acetylation seems to affect a number of dynamic cellular events, including the correct organization of the immune synapse (Serrador et al., 2004), the infection of CD4⁺ T cells by HIV1 (Valenzuela-Fernandez et al., 2005), the binding and transport of a kinesin 1 (Reed et al., 2006), and the dynamics of cellular adhesions (Tran et al., 2007).

Moreover, a stage-specific role for SIR2RP1 in *Leishmania* biology has been frequently proposed since: 1- the overexpression of SIR2rp1 led to an accumulation of the protein in the cytosol of both promastigotes and amastigotes, but an increase in parasite survival was only observed in the case of amastigotes (Vergnes et al., 2002); 2- the disruption of a single allele of the gene encoding the LiSIR2rp1 significantly affected the amastigotes' survival (Vergnes et al., 2005); 3- a commercially available inhibitor of Sirtuins: sirtinol, significantly inhibited the growth of *Leishmania* amastigotes (Vergnes et al., 2005). Is this stage-specific role linked to its enzymatic functions? Besides the enzyme co-factor and the enzymatic reaction products, what else might influence the LiSIR2RP1's enzymatic function? Furthermore, LiSIR2RP1 is only partially associated with the parasite's cytoskeleton. Consequently, the significant presence of this parasite protein in the detergent-soluble fraction of the parasite's total extract cannot be ignored and could be indicative of the existence of other substrates which might be involved in different biological processes.

2 Discovery of *Leishmania* specific SIR2 inhibitors

Even though it belongs to a conserved family of proteins that also exist in humans, we have considered LiSIR2RP1 as a potential drug target, mostly based on the facts that the deletion of one allele of the LiSIR2 locus is sufficient to dramatically affect amastigote proliferation, and that the sequence's homology with host SIR2 proteins is divergent enough. The successful inhibition of trypanosomal ornithine-decarboxylase by difluoromethylornithine (DMFO), which is used to treat African trypanosomiasis, is a common example that parasite selectivity can be achieved even when the target is also present in humans. Indeed, this specificity is due to such differences in the turnover rates of the human and parasite enzymes, that unable the parasite to regenerate enzyme fast enough to survive its irreversible blockade (reviewed in Burri and Brun, 2003).

In the search for target specific inhibitors, knowledge of the three-dimensional structure of a protein is of great importance. Fortunately, predictions of protein tertiary structure can be made, even in the absence of crystallographic structures, through the study of sequence-structure-function relationships. Indeed, until now, modelling by homology remains the most reliable method of predicting a protein's structure. Following this approach, a three-dimensional model of *L. major* SIR2 was constructed by Kadam and colleagues (2006), representing a reliable model of the

ligand binding domain, and shed new light on the binding features of this enzyme. Moreover, the analysis of LmSIR2 along with hSIRT2 molecular electrostatic potentials (MESP), cavity depth, and both models superposition suggested that the nicotinamide binding catalytic domain has several minor but potentially important structural differences that could be exploited for selective targeting (Kadam et al., 2006). Based on these subtle differences, an "*in silico*" screening of the National Cancer Institute compounds library was conducted, seeking the inhibition of the parasite SIR2 over the human enzyme. Although, this strategy failed to identify a truly potent and selective lead compound, it was effective in identifying N-(2-fluorophenyl) nicotinamide (molecule 56), which can be used for lead designing.

Some progresses have been made to identify Sirtuin inhibitors, even though they are not comparable with the ones made in the search for Class I and II HDAC inhibitors. Belonging to the hydroxamate, benzamide, aliphatic acids and cyclic peptides chemical classes, a number of derived molecules have reached clinical trials as anticancers (review in Xu et al., 2007). Moreover, the suberoyl anilide hydroxamic acid (SAHA) has already been approved by the Food and Drug Administration (FDA) to treat cutaneous T-cell lymphoma (Marks and Breslow, 2007). Generally, this type of inhibitors induces a variety of phenotypes in cancer cells, including growth arrest, activation of extrinsic and/or intrinsic apoptotic pathways, autophagic cell death, reactive oxygen species (ROS)-induced cell death, mitotic cell death and senescence. In comparison, normal cells are quite more resistant to cell death induced by these compounds (review in Xu et al., 2007). Moreover, the potential of Class I and II HDAC inhibitors against one of the agents of malaria, *P. falciparum*, have recently been elucidated. The fourteen derivatives designed to inhibit the PfHDAC1, based on the homology models of human class I and II HDAC, revealed to be very potent against the erythrocytic stage of the parasite (IC_{50} between 13-334nM) (Andrews et al., 2008). Furthermore, four genes encoding a class I and II human and yeast HDAC orthologues were identified in *T. cruzi*. Two of them were described as essential, and one required for normal cell cycle progression (Ingram and Horn, 2002). Are the parasite class I and II human HDAC orthologues potential drug targets?

The presence of a common structural moiety (naphthalene) in some molecules identified as Sirtuin inhibitors, like sirtinol, splitomycin and suramine, led us to discover BNIP derivatives as a new class of Sirtuin inhibitors. Surprisingly, these compounds were more potent inhibitors of LiSIR2RP1, when compared to its human counterpart. A strict correlation between the BNIP derivative structure and its inhibitory potency was only found in the parasite, and not in the human enzyme. Moreover, the most active and selective inhibitor (parasite versus human enzyme)

was the BNIPDanon. Indeed, based on kinetic studies, its inhibitory effect is due to competition with NAD^+ , which was later supported by the docking analysis of BNIP derivatives in the LiSIR2RP1 and hSIRT1 three-dimensional models. However, the two enzymes' binding mode differs considerably, which explains their different potencies. An outcome of this study has not only been the identification of a new class of Sirtuin inhibitors, but also the questioning of the existence of such (human versus parasite) differences in others NAD^+ -dependent enzymes. The use of NAD^+ from an organic chemistry point of view can be divided into three general categories. In the first category, NAD^+ is only used as a proton acceptor, and is therefore used by numerous enzymes involved in redox reactions. As an example, inosine 5'-monophosphate dehydrogenase (IMPDH) utilizes NAD^+ to oxidize inosine monophosphate into xanthosine monophosphate, a rate limiting step in the de novo synthesis of guanine nucleotides. In the second category, NAD^+ can be consumed, leading to the generation of nicotinamide, and products containing ADP-ribose. Multiple enzyme families, such as PARPs, Sirtuins, ADP-ribosyl cyclases and mono(ADP-ribosyl) cyclases, are included in this category. The third category involves further chemical or enzymatic modifications of the NAD^+ molecule. An example of this is the generation of nicotinamide adenine dinucleotide phosphate (NADP) by the phosphorylation of the 2' hydroxyl group on the adenosine ribose. Indeed, NAD^+ is involved in several cellular processes besides redox reactions, including signal transduction, DNA repair, and post-translational protein modifications, attracting medicinal chemists to the therapeutic potential of molecules affecting interactions of NAD^+ with NAD^+ -dependent enzymes, in diseases from cancer to aging (reviewed in, Chen et al., 2008 and Ying, 2008). Furthermore, the possible interaction of BNIP derivatives with enzymes or receptors that bind other adenosine-containing cofactors or ligands, such as kinases using ATP, cannot be excluded. Suramin and several related adenosine receptor antagonists inhibit sirtuins as well (Howitz et al., 2003). Moreover, a recent study has identified several new lead structures for Sirtuin inhibitors by searching a library of kinase and phosphatase inhibitors (Trapp et al., 2006). Therefore, two main questions arise concerning: the specificity of BNIP derivatives to Sirtuins; the possibility of achieving selective inhibition of an enzyme, by using molecules that interfere with enzyme co-factors.

3 BNIP derivatives and cisplatin as antileishmanial drugs

Our interest in the evaluation of the potential antileishmanial activity of BNIP derivatives has emerged with the first synthesized compounds, which contain polyamines such as putrescine, spermidine and spermine linking two naphthalimidopropyl moieties, expecting an interference with the parasites polyamines pathway. Indeed, polyamine biosynthesis is one area of parasite biology that has attracted attention in terms of drug development (Muller et al., 2001; Heby et al., 2007). The natural polyamines spermidine and spermine, and their precursor diamine putrescine, are present in most eukaryotic cells, playing pivotal roles in several cellular processes including protein/nucleic acid synthesis as well as cell proliferation/differentiation, and have been implicated in the development of certain cancers (reviewed in Gerner and Meyskins, 2004). Furthermore, in trypanosomatid protozoan parasites, polyamines have an additional role, since spermidine is used in the synthesis of trypanothione, a glutathione-spermidine dithiol conjugate that plays a central role in several detoxification processes and supplies reducing equivalents used in nucleic acid synthesis (Fairlamb et al., 1985; Fairlamb, 1992; Muller et al., 2003). As this thiol is both unique and essential to trypanosomatids, any process involving trypanothione is considered a potential target for drug intervention. Furthermore, the intracellular concentrations of polyamines in mammalian cells are regulated by feedback mechanisms involving multiple routes of synthesis and interconversion that differ from the parasite pathway (reviewed in Muller, 2001). The polyamine derivative compounds, namely BNIPPut, BNIPSpd and BNIPSpm, were effective in reducing the *L. infantum* promastigotes and axenic amastigotes' growth in a dose-dependent manner. The compound containing the smallest polyamine chain, BNIPPut, was found to be the most potent with IC₅₀ values of 1.30 and 1.68 μM for promastigotes and axenic amastigotes, respectively. Indeed these drugs were capable of inducing promastigotes' cell death, whose phenotype shares extensive homology with metazoan apoptosis, including DNA fragmentation, phosphatidylserine externalization and mitochondria transmembrane potential loss, but failed to do so in the case of axenic amastigotes. Furthermore, the anticancer drug cisplatin has also exerted antileishmanial activity *in vitro*, being more effective in the reduction of the parasite's vertebrate stage growth. Even though it induces mitochondrial transmembrane potential loss in both parasite forms, DNA fragmentation was only

detected in the promastigote stage. Therefore, both studies suggest that the mechanisms leading to the parasite's growth arrest are different according to the parasite's stage. The elucidation of the molecular events which tightly regulate the processes by which these compounds induce *Leishmania*'s growth arrest and death might allow the definition of a more comprehensive view of the operating cell's death machinery. It was initially assumed that apoptosis arose with multicellularity, being crucial for embryogenesis, tissue homeostasis and disease control (Vaux and Strasser, 1996). However, several findings have indicated the occurrence of a similar process in single celled eukaryotes, including kinetoplastid protozoan parasites (Ameisen et al., 1995; Moreira et al., 1996; Das et al., 2001; Arnoult et al., 2002; Mukherjee et al., 2002). Indeed, it has been proposed that programmed cell death (PCD) through apoptosis may occur in unicellular organisms to control the cell population by: selecting the fitter cells within the population; optimally regulating the cell number to adapt to environmental constraints; tightly controlling the cell cycle and differentiation; and maintaining clonality within the population (Lee et al., 2002). Moreover, various types of stimulus induce in *Leishmania* some features similar to those of mammalian caspase-dependent apoptosis, including nuclear chromatin fragmentation, internucleosome-like DNA fragmentation, phosphatidylserine exposure on the outer leaflet of the plasma membrane, mitochondrial permeabilization and loss of transmembrane potential (Arnoult et al., 2002; Kulkarni et al., 2006). Nevertheless, genome database comparisons revealed that the majority of proteins involved in mammalian apoptosis (especially from the Bcl-2/Bax pathway) are apparently not encoded in the genome of *Leishmania* or related protozoa (Ivens et al., 2005). Significantly, no caspase encoding gene can be found in the two completed *Leishmania* genomes described so far. Indeed, two *L. donovani* metacaspases have recently been characterized. These enzymes, containing a C-terminal proline rich domain, were unable to cleave caspase-specific substrates but efficiently cleave trypsin substrates (Lee et al., 2007). Unlike what was expected, metacaspases do not seem to be responsible for the caspase-like activity already reported in *Leishmania*, even though they might have a role in *Leishmania* apoptosis, as suggested by their increased activity upon cell treatment with H_2O_2 and increased susceptibility to H_2O_2 in the cells overexpressing metacaspases (Sen et al., 2004; Singh et al., 2005; Lee et al., 2007).

The antileishmanial and anticancer activities exerted by the polyamine containing BNIP derivatives led to the synthesis of several others derivatives, whose central chain was targeted for diversity. All compounds were tested against *L. infantum* promastigotes, axenic and intracellular amastigotes. Indeed, despite the usefulness of axenic amastigotes in drug screening, it is not possible to ignore that, ideally, the

drugs' effectiveness against *Leishmania* should be determined using intracellular amastigotes. Moreover, differences between axenic and intracellular amastigotes' sensitivity to some BNIP derivatives were also reported, reinforcing the necessity of evaluating drugs' activity against intracellular amastigotes. Furthermore, some BNIP derivatives exhibited antileishmanial activity in mice model of VL, without signals of acute toxicity in the blood, at least. However, although effective in reducing the parasite load, BNIP derivatives were unable to eliminate all *Leishmania* parasites from the infected animals. Therefore, additional experiments will be required to further improve BNIP derivatives' *in vivo* efficacy.

4 New therapeutic options against VL: what is really needed?

With the exception of southern Europe, the access to ready diagnosis, affordable treatment and effective disease control is limited due to economic reasons. The necessity of improving the drug repertoire to treat leishmaniasis is commonly agreed. Among some of the reasons are the severe toxicity, the emergence of resistances and the limited efficacy of the recommended therapy mostly in the case of immune-compromised individuals (HIV infection). The introduction of amphotericin B lipid formulations for the treatment of VL has been noteworthy, since it enables a significant reduction in the occurrence of toxic effects and therapeutic duration. Unfortunately, its use in endemic areas is blocked due to economic reasons. Therefore, we consider that overcoming this situation is extremely urgent, in order to guarantee the availability of an effective therapy in endemic areas. However, despite the success of such amphotericin B formulations, lack of effectiveness has been reported in the case of immune-compromised individuals, such as HIV/*Leishmania* co-infected patients, where relapses occur frequently (Sundar et al., 2002; Meyerhoff et al., 1999). Greater attention should be paid to this situation, since it might contribute to the selection of resistant parasites. Furthermore, it is questionable whether what we call effective drugs promote an overall clearance of the parasites in infected patients. Indeed, it is possible that a few number of *Leishmania* parasites could persist in cured individuals and their progress controlled by an effective immune system. In the case of immune-compromised individuals, however, the parasites progression may occur, leading to disease. Therefore, an exploitation of potential parasite persistence sites upon an effective drug treatment might disclose unravelled locations where drugs probably do not but should have access.

Chapter VII

Publications in related fields

A *Leishmania infantum* cytosolic tryparedoxin activates B cells to secrete interleukin-10 and specific immunoglobulin

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Summary

The immune evasion mechanisms of pathogenic trypanosomatids involve a multitude of phenomena such as the polyclonal activation of lymphocytes, cytokine modulation and the enhanced detoxification of oxygen reactive species. A trypanothione cascade seems to be involved in the detoxification process. It was recently described and characterized a tryparedoxin (*LiTXN1*) involved in *Leishmania infantum* cytoplasmatic hydroperoxide metabolism. *LiTXN1* is a secreted protein that is up-regulated in the infectious form of the parasite, suggesting that it may play an important role during infection. In the present study, we investigated whether recombinant *LiTXN1* (*rLiTXN1*) affects T- and B-cell functions in a murine model. We observed a significant increase in the CD69 surface marker on the B-cell population in total spleen cells and on isolated B cells from BALB/c mice after *in vitro* *rLiTXN1* stimulus. Activated B-cells underwent further proliferation, as indicated by increased [³H]thymidine incorporation. Cytokine quantification showed a dose-dependent up-regulation of interleukin (IL)-10 secretion. B cells were identified as a source of this secretion. Furthermore, intraperitoneal injection of *rLiTXN1* into BALB/c mice triggered the production of elevated levels of *rLiTXN1*-specific antibodies, predominantly of the immunoglobulin M (IgM), IgG1 and IgG3 isotypes, with a minimum reactivity against other heterologous antigens. Taken together, our data suggest that *rLiTXN1* may participate in immunopathological processes by targeting B-cell effector functions, leading to IL-10 secretion and production of specific antibodies.

Keywords: *Leishmania infantum*; *LiTXN1*; B cells; interleukin-10; specific antibodies

Introduction

Leishmaniasis, a disease caused by protozoan parasites of the genus *Leishmania*, remains a serious public health problem in areas of the tropics, the subtropics and southern Europe, with approximately 12 million people being affected in these regions.¹ Depending on the interaction between the infecting *Leishmania* species and the immune status of the host, the clinical manifestations can vary from self-healing cutaneous ulceration to progressive visceral dissemination, which is fatal if untreated.²

Leishmania are intracellular parasites that reside primarily within host tissue macrophages and dendritic cells. Interestingly, these cells participate in the first line of defence against pathogens through their antimicrobial and/or antigen-presenting functions.³ In murine models of experimental leishmaniasis, the development of potent type-1 CD4⁺ cell-mediated immunity was determined to be the general protective mechanism against all *Leishmania* species. This will ultimately lead to parasite destruction by interferon (IFN)- γ stimulated macrophages through tumour necrosis factor (TNF)- α and nitric oxide

Abbreviations: ConA, concanavalin A; FCS, fetal calf serum; i.p., intraperitoneally; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; *rLiTXN1*, recombinant *Leishmania infantum* tryparedoxin 1.

(NO)-dependent mechanisms.³ *Leishmania* has developed several strategies to escape the host immune system in order to successfully establish an infection. For instance, *Leishmania* parasites are capable of modelling the T-cell response and cytokine production towards a non-protective T helper type 2 (Th2) response, characterized by the secretion of anti-inflammatory cytokines such as interleukin (IL)-4, IL-10, IL-13 and transforming growth factor (TGF)- β .^{4–6} In addition to the suppression of parasite specific Th1 cell-mediated responses during active disease, a marked increase in the humoral response is induced, which is characterized by the secretion of large amounts of parasite non-specific antibodies with self autoreactivity, particularly of the immunoglobulin M (IgM) and IgG isotypes.⁷ To date, several *Leishmania* antigens involved in these pathological pathways have been identified. It was shown that a single T-cell epitope derived from the *Leishmania* homologue of receptor for activator C kinase (LACK) antigen was responsible for early IL-4 production, which is critical for Th2 differentiation, by the V β 4 V α 8 CD4⁺ T-cell population in BALB/c mice, contributing to the development of progressive disease in these mice.⁸ Moreover, we have previously reported the identification of a *Leishmania major* protein, homologous to the mammalian ribosomal protein S3a, which participates in the immunoregulatory process by inducing polyclonal expansion of non-specific, non-parasite directed B-cell clones and suppression of Th1-type cytokine production.⁹ Also, other antigens have the capacity to polarize the immune response towards a Th2 phenotype, thus exacerbating the disease; for example, lipophosphoglycan in *L. major*¹⁰ and papLE22 in *Leishmania infantum*¹¹ do so by reducing the levels of inflammatory cytokines IL-1 and TNF- α on stimulated macrophages and by increasing the level of the immunosuppressive cytokine IL-10, respectively. Thus, it has been argued that it is of critical importance to characterize parasite antigens that potentiate the disease, as an effective vaccine should be capable of preventing the action of these 'immunopathological' antigens.¹¹

As the major *Leishmania* species complexes diverged some 40–80 million years ago,¹² it is not surprising that different correlatives of protection are found for each pathology. For example, in chronic murine visceral leishmaniasis, while TGF- β has been shown to inhibit the Th1-associated resolution of infection,¹³ IL-4 has been shown not to contribute to the disease outcome.¹⁴ Nevertheless, there is considerable evidence supporting a central immunosuppressive role for the endogenous IL-10.¹⁵ Antigen-induced production of IL-10 is of major interest because of the antagonistic effects of IL-10 on IFN- γ .¹⁶ This Th1 suppressive cytokine is also responsible for compromising antigen specific T-cell stimulation and for impairment of macrophage activation.¹⁶ Thus, IL-10 is generally considered to be the major cytokine involved in the progression to visceral disease.¹⁷

In a previous study, we identified a cytosolic *L. infantum* trypanoredoxin (*LiTXN1*) that belongs to a special class of oxidoreductases found in trypanosomatids and that is related to the ubiquitous thioredoxins. *LiTXN1* is expressed throughout the parasite life cycle.¹⁸ However, it was observed to be up-regulated and excreted/secreted only in the infective stages.¹⁹

The purpose of our study was to characterize the effect of this secreted protein on the immune system, focusing on T- and B-cell functions. The results showed that *rLiTXN1* is a potent B-cell activator in the presence or absence of T lymphocytes. The activated B cells underwent both proliferation and differentiation, as shown by the production of specific antibodies. However, it also promoted the B-cell production of IL-10, suggesting a potential role in disease progression.

Materials and methods

Mice

Six-week-old male BALB/c mice were obtained from Harlen Iberica (Barcelona, Spain) and 6-week-old male C3H/HeJ mice were purchased from Charles River (L'Arbresle, France).

Purification of *rLiTXN1*

The *L. infantum* TXN1 protein was produced from pre-transformed *Escherichia coli* BL21 clones.¹⁸ The protein used was obtained as a recombinant protein (*rLiTXN1*) containing six histidine residues at its N-terminal. *rLiTXN1* purity was determined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and staining with Coomassie blue. For biological assays, the protein was dialysed against phosphate-buffered saline (PBS) using PD10 desalting columns (Amersham, Arlington, IL). The recombinant protein content was determined using the Folin procedure.²⁰

Injection of mice

Six- to seven-week-old BALB/c mice were injected three times intraperitoneally (i.p.), at 7-day intervals, with 50 μ g of *rLiTXN1*. Control mice were injected with PBS. Two weeks after the final injection, spleens and sera were collected.

Spleen B-cell isolation

After cervical dislocation, spleens were removed and homogenized to obtain a single cell suspension. The cells were washed two times in RPMI-1640 culture medium (Bio-Wittaker, Verviers, Belgium) and adjusted to 10⁷ cells/ml in RPMI-1640 culture medium supplemented with

10% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 20 mM HEPES. Spleen B cells were isolated by depletion of non-B cells using the B-cell isolation kit MACS® (Miltenyi Biotec, Auburn, CA) following the manufacturer's instructions. The purity of enriched B cells was evaluated by flow cytometry, using double labelling with specific monoclonal antibodies (mAb; anti-µ⁺, anti-CD4⁺ and anti-CD8⁺). B-cell purity above 97% was obtained after the purification process.

Cell culture and proliferation assays

Single cell suspensions (2×10^5 cells/well) of spleen cells or isolated B cells, from normal or r*LtTXN1* injected BALB/c mice or from normal C3H/HeJ mice, were cultured in 96-well flat-bottom plates at a final volume of a 200 µl. Cells were stimulated with concanavalin A (ConA; 6 µg/ml), lipopolysaccharide (LPS; 10 µg/ml) or r*LtTXN1* (5, 10 or 50 µg/ml) and incubated at 37° in 5% CO₂. On days 1, 2 and 3 of culture, the cells were pulsed with 1 µCi of [³H]thymidine (Amersham) for the last 8 hr. Pulsed splenocytes were harvested on a glass filter using an automated multiple sample harvester and dried. Incorporation of radioactive thymidine was then determined by liquid scintillation as specified in a standard protocol. Assays were performed in triplicate, and the stimulatory index was calculated by dividing the arithmetic mean counts per minute (c.p.m.) obtained from stimulated cultures by the arithmetic mean c.p.m. obtained from control cultures without stimulation.

Flow cytometry

Spleen cells or isolated B cells from normal or r*LtTXN1*-injected BALB/c mice obtained as single cell suspensions were washed in PBS supplemented with 2% FCS. A total of 10^6 viable cells were incubated for 30 min at 4° with saturating concentrations of phycoerythrin (PE)-conjugated hamster anti-mouse CD69 plus fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD8 (Ly-2) monoclonal antibody, FITC-conjugated rat anti-CD4, or FITC-conjugated goat anti-mouse IgM (BD Pharmingen, San Diego, CA). After two washes with PBS/2% FCS, the cells were analysed by flow cytometry in a FACScan equipped with CELLQUEST PRO software (Becton Dickinson, San Jose, CA). Dead cells were excluded from all samples by propidium iodide labelling.

Enzyme-linked immunosorbent assays (ELISAs) for cytokines

Cytokine production was determined by two site sandwich ELISAs in the supernatants of spleen cells or isolated B cells from BALB/c mice, stimulated with ConA (6 µg/ml),

LPS (10 µg/ml) or r*LtTXN1* (5 or 50 µg/ml) and collected after 24 hr (IL-2), 48 hr (IL-10 and IL-4) or 72 hr (IFN-γ) of incubation at 37° and 5% CO₂. Ninety-six-well flat-bottom microtitre plates (Maxisorp Immuno-plates; Nunc, Roskilde, Denmark) were coated overnight at 4° with unlabelled rat antibodies to the cytokines IL-2 (JES6-1A12 cell line), IL-4 (BVD4-1D11 cell line), IFN-γ (R4-6A2 cell line) and IL-10 (Mouse IL-10 ELISA Set from BD Pharmingen, San Diego, CA). IL-10 measurement was performed following the manufacturer's instructions. For the others ILs, the plates were washed with PBS/0.1% Tween-20 (PBS-T) and blocked with PBS/1% gelatine for 1 hr at room temperature. After 2 hr of incubation with serial dilutions of each supernatant, plates were washed with PBS-Tween 20 and incubated for another 1 hr with biotinylated rat antibodies to IL-2 (JES6-5H4 cell line), IL-4 (BVD6-24G2 cell line) and IFN-γ (XMGI-2 cell line), from BD Pharmingen. The plates were then washed with PBS-T and incubated for 1 hr with streptavidin-peroxidase (Sigma, St Louis, MO) and developed with 0.5 mg/ml of *o*-phenylenediamine dihydrochloride (OPD; Sigma) and H₂O₂ in citrate buffer. Reactions were stopped by the addition of 10% SDS. Optical densities were measured at 450 nm in an automatic ELISA reader (Power WaveTMXS; Bio-Tek, Winooski, VT). Cytokine concentrations were determined by comparison with a standard curve generated with the different recombinant cytokines: rIL-2, rIL-4 and rIFN-γ (BD Pharmingen).

ELISA for immunoglobulins

Ninety-six-well flat-bottom plates (Greiner Labortchnik, Solingen, Germany) were coated overnight at 4° in carbonate buffer, pH 8.2, with unlabelled goat anti-mouse immunoglobulin (5 µg/ml), total *L. infantum* protein extract (10 µg/ml), r*LtTXN1* (5 µg/ml), recombinant *L. infantum* cytosolic trypanredoxin peroxidase (r*LicTXNPx*; 5 µg/ml), bovine serum albumin (BSA; 5 µg/ml), whale skeletal muscle type II myoglobin (Myo; 5 µg/ml), or native double stranded DNA (DNA; 5 µg/ml). The plates were washed with PBS-T and blocked with PBS/1% gelatine for 1 hr at 37°. Plates were then incubated for 2 hr with serial dilutions (for total titres) or 1 : 900 dilutions (for specific antibody reactivity) of each serum sample (from PBS or r*LtTXN1* injected mice). After washing with PBS-T, the plates were incubated for 30 min at 37° with peroxidase-labelled goat anti-mouse immunoglobulin isotypes (anti-IgM, anti-IgG, anti-IgG1, anti-IgG2a, anti-IgG2b and anti-IgG3) and developed with 0.5 mg/ml OPD (Sigma) and H₂O₂ in citrate buffer. Reactions were stopped by the addition of 3 N HCl. Unlabelled purified isotypes were used in serial dilutions as standards. Absorbance values were read at 492 nm in an automatic ELISA reader (Power WaveTMXS).

Reverse transcription–polymerase chain reaction (RT-PCR)

Isolated spleen B cells were stimulated or not *in vitro* with rLiTXN1 (5 and 50 µg/ml) and incubated at 37° in 5% CO₂. After 12 hr of stimulation, total RNA (from 4×10^6 cells per stimulus) was isolated using a guanidium thiocyanate-phenol-chloroform single step method.²¹ cDNA synthesis was achieved with 1 µg of total extracted RNA using Superscript II RT (Gibco BRL, New York, NY) and random primers (Promega, Madison, WI). PCR amplification was performed with 1 µg of cDNA using primers for IL-10 and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). After 35 PCR cycles consisting of DNA denaturation at 94° for 30 seconds, primer annealing at 55° for 30 seconds, and DNA extension at 72° for 30 seconds, the reaction was terminated. Then 25 µl of each PCR product was analysed by electrophoresis and visualized and photographed on a UV transilluminator (Vilber Lourmat, Eberhardzell, Germany). PCR band densities were determined using BIO-POFIL BIO-1D software (Vilber Lourmat).

Western blot

Parasite cell lysates (30 µg/lane) or rLiTXN1 (2 µg/lane) was electrophoresed on 15% SDS-PAGE gels and transferred onto a nitrocellulose membrane. After saturation for 1 hr with a PBS solution containing 5% fat dehydrated milk, the membranes were incubated with different dilutions of polyclonal sera against rLiTXN1 and sera from control mice (PBS injected mice) overnight at 4°. After washing with PBS-T, the membranes were incubated with horseradish peroxidase (HRP)-labelled goat anti-mouse IgG antibody (Southern Biotechnologies, Birmingham, AL) for 1 hr and revealed with the ECL western blotting analysis system (Amersham).

Statistical analysis

The data were analysed using Student's *t*-test.

Results

rLiTXN1 induces CD69 expression in murine B cells

In a previous study, we examined the expression pattern of LiTXN1 during the *Leishmania* life cycle and found it to be up-regulated during the stationary promastigote phase and the intracellular amastigote stage of the parasite.¹⁸ Moreover, in these stages, LiTXN1 is abundantly excreted/secreted from the parasite.¹⁹ Thus, we decided to examine the effect of LiTXN1 on the host immune system cells. Hence, we subcloned the LiTXN1 encoding gene into an expression vector and the corresponding recombinant protein (rLiTXN1) was purified.

To identify the cell populations responding to rLiTXN1, we searched for the cell membrane expression of an early activation marker (CD69) in CD4⁺, CD8⁺ and B-cell populations after 20 hr of *in vitro* rLiTXN1 stimulation (Fig. 1a). The fluorescence-activated cell sorting (FACS) analysis showed a dose-dependent increase in CD69⁺ B cells after rLiTXN1 stimulation ($12.2 \pm 1.2\%$ (mean $\pm$ standard deviation) and $28.8 \pm 0.44\%$ positive B cells upon stimulation with 5 and 50 µg/ml of rLiTXN1, respectively) compared with unstimulated cells ($4.3 \pm 1.4\%$ positive B cells). However, no significant differences were found for the CD4⁺ and CD8⁺ T-cell populations. As a positive control we used a known B-cell mitogen, LPS, which induces high expression of CD69 on B cells. In order to rule out the possibility that rLiTXN1 biological activity resulted from contaminating bacterial components present in our recombinant protein, additional experiments were performed with the addition of polymyxin B. In these conditions, the LPS activity was

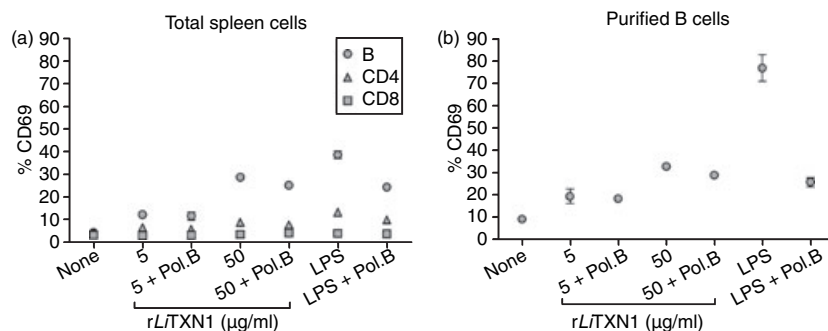


Figure 1. Recombinant *Leishmania infantum* trypanodioxin 1 (rLiTXN1) induces the expression of CD69 on total spleen and purified B cells from BALB/c mice. Expression of the CD69 activation marker in total spleen cells (a) and purified B cells (b) from BALB/c mice after *in vitro* stimulation with rLiTXN1 is shown. Cells (2.5×10^5 per well) were cultured in the presence of rLiTXN1 (5 or 50 µg/ml) or lipopolysaccharide (LPS) (10 µg/ml), both with and without 10 µg/ml of polymyxin B (Pol.B). After 20 hr, percentages of CD69⁺ B cells and CD4⁺ and CD8⁺ T cells were determined by flow cytometry analysis. The results represent the means and standard deviations of three experiments carried out independently.

partially abrogated, while no significant differences were observed in B-cell activation induced by *rLiTXN1*.

Furthermore, to elucidate whether T-cell dependent mechanisms are involved in the B-cell activation induced by *rLiTXN1*, we purified resting B cells from naïve BALB/c splenocytes. As shown in Fig. 1(b), the *rLiTXN1* stimulus induced a significant increase in CD69 expression on isolated B cells ($19.5 \pm 3.2\%$ and $32.3 \pm 0.9\%$ positive B cells upon stimulation with 5 and 50 $\mu\text{g/ml}$ of *rLiTXN1*, respectively) when compared with the unstimulated cells ($9.2 \pm 1.2\%$ positive B cells). Similar results were obtained when polymixin B was added to *rLiTXN1*-treated B cells, suggesting that the observed effects are a result of *rLiTXN1* biological activity and not bacterial contamination. Thus, it is reasonable to assume that *rLiTXN1* behaves as a B-cell activator, in the presence or absence of T cells.

In vitro effects of *rLiTXN1* on B-lymphocyte proliferation

To further examine the effect of *rLiTXN1* on spleen cell populations, we measured proliferation using the incorporation of [^3H]thymidine in total spleen and isolated B cells submitted to *in vitro* *rLiTXN1* stimulus. Spleen cells from BALB/c mice were cultured in the presence or absence of *rLiTXN1* for 48 and 72 hr. As shown in Fig. 2(a), total spleen cells from BALB/c mice responded in a dose dependent manner to *rLiTXN1* *in vitro* stimulus, as revealed by increased thymidine incorporation compared with unstimulated cells.

These results suggest that B cells are a target for *rLiTXN1*. B-cell proliferation can occur in a T-cell dependent or independent manner. We found that, even in the absence of a T-cell population, isolated resting B cells proliferated in response to *rLiTXN1* *in vitro* stimulus (Fig. 2b). Moreover, the *rLiTXN1* induced proliferation was significantly enhanced in isolated B cells compared with the results obtained for total spleen cells, as confirmed by an increased stimulatory index (32.62 versus

Table 1. Stimulation index for total spleen cells and purified spleen B cells from BALB/c mice, stimulated *in vitro* with recombinant *Leishmania infantum* trypanodoxin 1 (*rLiTXN1*) (50 $\mu\text{g/ml}$)

	Total spleen cells	Purified B cells	48 hr	72 hr
LPS	+	–	89.93	97.66
	–	+	93.65	100.15
<i>rLiTXN1</i>	+	–	10.98	16.63
	–	+	27.93	32.62

LPS, lipopolysaccharide.

16.63 after 72 hr of stimulation) (Table 1). In order to rule out the possibility that LPS contaminating the *rLiTXN1* preparation was responsible, at least in part, for the cell stimulation, spleen cells from C3H/HeJ mice (LPS non-responder) were cultured with or without *rLiTXN1*. The proliferation of C3H/HeJ spleen cells induced by *rLiTXN1* stimulus reached similar levels to those observed with BALB/c cells, as revealed by the corresponding stimulatory index: 2.42, 5.96 and 9.09 for C3H/HeJ mice and 2.57, 6.64 and 10.98 for BALB/c mice after 48 hr of stimulation with 5, 10 and 50 $\mu\text{g/ml}$ of *rLiTXN1*, respectively. These observations suggest that B cells are a putative *rLiTXN1* target.

rLiTXN1 up-regulates IL-10 production in spleen cells

Complementary investigations were performed for the cellular responses induced by *rLiTXN1*, namely cytokine production. Thus, spleen cells from BALB/c mice were cultured *in vitro* in the presence or absence of ConA, as a positive control, or *rLiTXN1*, and the supernatant levels of IL-2, IL-4, IL-10 and IFN- γ were measured by ELISA. As shown in Table 2, a significant dose-dependent increase in IL-10 secretion by spleen cells stimulated with *rLiTXN1* was detected compared with unstimulated cells ($P < 0.01$ for 5 and 50 $\mu\text{g/ml}$ of *rLiTXN1*). No significant variations were observed for

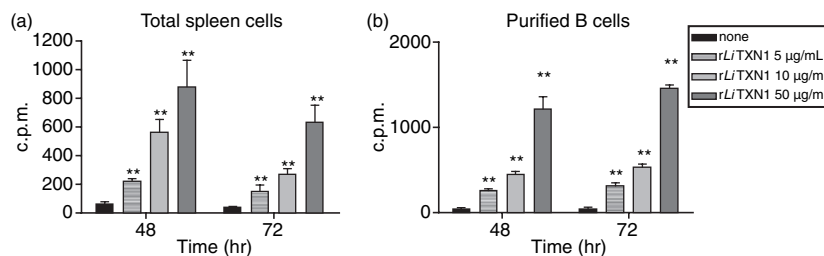


Figure 2. Lymphocyte proliferation of BALB/c mice spleen cells induced by recombinant *Leishmania infantum* trypanodoxin 1 (*rLiTXN1*). Total spleen cells (a) or purified spleen B cells (b) (2.5×10^5 cells per well) from BALB/c mice were stimulated *in vitro* with increasing concentrations of *rLiTXN1* (5, 10 or 50 $\mu\text{g/ml}$) for 48 or 72 hr. Cells were pulsed with [^3H]thymidine for the last 8 hr. Data [counts per minute (c.p.m.)] represent the mean and standard deviation for three animals analysed individually and are representative of three independent experiments. Statistically significant differences between non-stimulated and stimulated cells are indicated: ** $P < 0.01$.

Table 2. Increase in interleukin (IL)-10 production by spleen cells from BALB/c mice in response to recombinant *Leishmania infantum* trypanredoxin 1 (rLiTXN1) stimulation

Cytokine	rLiTXN1 treatment	Cytokine level (ng/ml) in supernatants ¹ of spleen cells from untreated and rLiTXN1 treated BALB/c mice			
		Control	ConA (5 µg/ml)	rLiTXN1 (5 µg/ml)	rLiTXN1 (50 µg/ml)
IFN-γ	–	1.66 ± 0.32	7.45 ± 0.77	1.58 ± 0.50	1.48 ± 0.25
	+	2.16 ± 0.44	9.08 ± 2.54	1.76 ± 0.59	2.85 ± 1.01
IL-2	–	0.26 ± 0.05	5.09 ± 0.27	0.22 ± 0.03	0.21 ± 0.05
	+	0.27 ± 0.05	5.48 ± 1.01	0.25 ± 0.02	0.23 ± 0.04
IL-10	–	0.03 ± 0.00	1.05 ± 0.09	0.16 ² ± 0.04	0.58 ² ± 0.19
	+	0.04 ± 0.02	1.26 ± 0.20	0.21 ² ± 0.05	0.76 ² ± 0.09
IL-4	–	0.22 ± 0.05	1.08 ± 0.11	0.23 ± 0.05	0.16 ± 0.05
	+	0.27 ± 0.09	1.27 ± 0.24	0.20 ± 0.05	0.20 ± 0.04

¹The cytokine levels in the supernatants of spleen cells (2.5×10^5 cells per well) from untreated or rLiTXN1 treated BALB/c mice were cultured with concanavalin A (ConA) or rLiTXN1 for 24 hr (IL-2), 48 hr (IL-4 and IL-10) or 72 hr [interferon (IFN)-γ]. These levels were determined by enzyme-linked immunosorbent assay (ELISA) in comparison with a standard curve using the recombinant interleukins. The data represent the means and standard deviations for three animals analysed individually and are representative of three independent experiments.

²Significant differences ($P < 0.01$) in IL-10 levels were observed between non-stimulated and rLiTXN1-stimulated cells, from both untreated and rLiTXN1 treated BALB/c mice.

IL-2, IL-4 and IFN-γ secretion after rLiTXN1 stimulus. Moreover, we determined the effect of *in vivo* rLiTXN1 treatment on *ex vivo* cytokine secretion by spleen cells after ConA or rLiTXN1 restimulation. Total spleen cells from rLiTXN1-treated mice also increased IL-10 secretion when restimulated with rLiTXN1. However, no significant differences were found between the IL-10 production by spleen cells of rLiTXN1 treated and untreated mice after rLiTXN1 *ex vivo* stimulus, suggesting that no B-cell memory was created.

B cells secrete IL-10 after rLiTXN1 stimulus

B cells are one of the cellular sources of IL-10.²² Thus, given that rLiTXN1 triggered B-cell activation, we examined the splenic B-cell population as a possible source of the IL-10 production induced by the rLiTXN1 stimulus. RT-PCR analysis was performed using specific primers for IL-10 and GAPDH, as an internal control, with mRNA purified from isolated B cells after incubation for 12 hr with or without rLiTXN1. rLiTXN1 stimulus induced a 1.3 fold increase (1.32 ± 0.07 ; $P < 0.01$) in IL-10 mRNA expression compared with the non-stimulated cells. Because IL-10 mRNA expression was increased, we measured IL-10 secretion by isolated B cells after 48 hr of rLiTXN1 stimulation. In these conditions, we detected a dose-dependent increase in IL-10 concentration in the supernatant of isolated B cells compared with unstimulated cells ($P < 0.01$ for 5 and 50 µg/ml of rLiTXN1) (data not shown). These observations suggest that B cells are involved in IL-10 secretion in response to the rLiTXN1 stimulus.

rLiTXN1 injection promotes a specific humoral response

In order to explore the *in vivo* effects of rLiTXN1 on the B-cell response, we immunized BALB/c mice i.p. without adjuvant, as described in the Materials and methods. Mice injected with PBS were used as a control group. Although rLiTXN1 induced B-cell activation and proliferation *in vitro*, we did not find significant increases in the spleen B-cell population after *in vivo* rLiTXN1 treatment (data not shown). However, a strong humoral response was developed in mice that received the rLiTXN1 immunization compared with control PBS-injected mice. As shown in Table 3, total serum levels of both IgM and IgG were found to be significantly increased (1.5 fold, $P = 0.01$, and 2.7 fold, $P < 0.001$, respectively) in the rLiTXN1-treated mice compared with the untreated mice, as determined by ELISA, 15 days after the last i.p. admin-

Table 3. Total serum immunoglobulins in BALB/c mice, 14 days after recombinant *Leishmania infantum* trypanredoxin 1 (rLiTXN1) administration

BALB/c	Total serum immunoglobulin (mg/ml)	
	IgM	IgG
PBS-treated	0.85 ± 0.11	1.22 ± 0.23
rLiTXN1-treated	1.28 ± 0.23	3.33 ± 0.25
	$P = 0.01$	$P = 1 \times 10^{-6}$

PBS, phosphate-buffered saline.

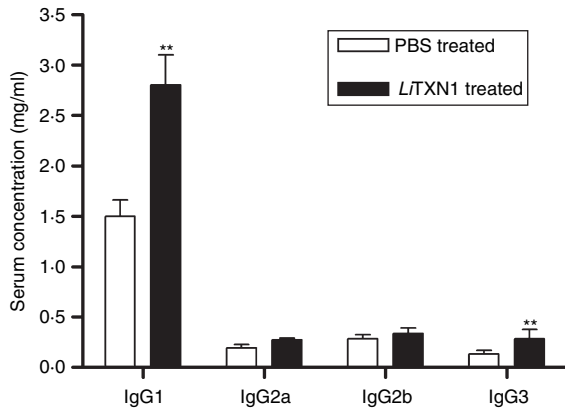


Figure 3. The humoral immune response induced by the intraperitoneal (i.p.) administration of recombinant *Leishmania infantum* trypanodoxin 1 (rLiTXN1) in BALB/c mice. Titres of immunoglobulin G (IgG) isotypes (IgG1, IgG2a, IgG2b and IgG3) in the sera of BALB/c mice were determined by enzyme-linked immunosorbent assay (ELISA), 14 days after the last rLiTXN1 i.p. administration (50 µg/mouse). The results are the arithmetic means of three animals analysed individually and are representative of three independent experiments. Statistically significant differences in IgG1 and IgG3 levels were observed between sera from rLiTXN1-treated and control mice: ** $P < 0.01$.

istration. Moreover, analysis of IgG antibodies showed that only IgG1 (1.9 fold, $P < 0.01$) and IgG3 (2.2 fold, $P < 0.01$) serum levels were significantly increased in immunized mice compared with controls (Fig. 3).

Further, we tested the specificity of these antibodies. The levels of rLiTXN1 specific serum antibodies of all tested isotypes were increased in previously immunized mice. However, this increase was higher for IgG1 and IgG3 than for the other isotypes studied (data not shown). In addition, no significant reactivity was found against heterologous antigens (i.e. Myo, BSA and DNA) or another recombinant *Leishmania* excreted/secreted protein, namely *Leishmania infantum* cytosolic trypanodoxin peroxidase (LicTXNPx), in rLiTXN1 treated mice (Fig. 4). Moreover, a significant increase in *L. infantum* total extract IgG levels was found in an ELISA test (data not shown). This is not surprising given that trypanodoxin proteins are known to occur in high amounts in the cell.²³ However, to confirm the specificity of rLiTXN1 treated mice serum among all parasitic antigens, we analysed by western blot their reactivity against the *Leishmania* total protein extract. Sera from mice immunized with rLiTXN1 recognized specifically the corresponding antigen (molecular weight approximately 16.6 kDa) in *L. infantum* protein extracts, whereas no reactivity was found for serum from PBS-injected mice (data not shown). Thus, rLiTXN1 when injected into BALB/c mice is capable of inducing B-cell differentiation leading to a specific humoral response.

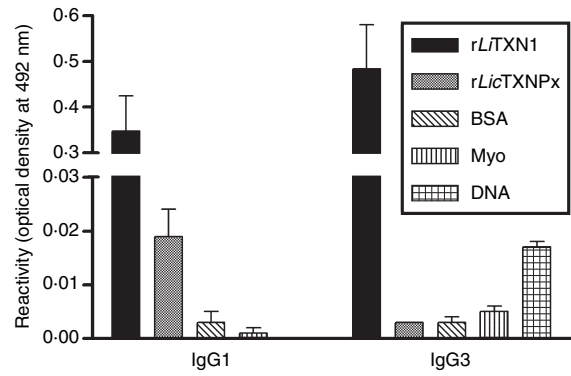


Figure 4. A specific humoral immune response is induced by recombinant *Leishmania infantum* trypanodoxin 1 (rLiTXN1) intraperitoneal (i.p.) administration in BALB/c mice. Immunoglobulin G1 (IgG1) and IgG3 antibody levels in the sera of rLiTXN1 treated BALB/c mice reacting against rLicTXNPx, bovine serum albumin (BSA), whale skeletal muscle type II myoglobin (Myo) and native double-stranded DNA (DNA) were determined by enzyme-linked immunosorbent assay (ELISA). Mice sera were diluted 1/900 and optical density was recorded at 492 nm. The data represent the means and standard deviations for three mice analysed individually and are representative of three independent experiments.

Discussion

Leishmania is a typical intracellular pathogen. Shortly after inoculation in the dermis, metacyclic promastigotes infect mononuclear phagocytic cells in the skin and differentiate intracellularly into non-motile amastigotes. For successful establishment of the infection, *Leishmania* must be capable of surviving the acidic and protease rich environment inside the phagolysosome but also deal with oxygen and nitrogen reactive species (ROS and RNS, respectively), which form part of the host antimicrobial machinery. We have recently identified and cloned a novel *L. infantum* gene that encodes a cytosolic trypanodoxin homologue to previously known trypanodoxins of *Crithidia fasciculata*, *Trypanosoma brucei* and *Trypanosoma cruzi*. It was suggested that LiTXN1 may be implicated in the resistance to ROS and RNS, as it is a probable component of parasite cytoplasmatic hydroperoxide metabolism.¹⁸ LiTXN1 is expressed throughout the parasite life cycle. However, northern and western blot analysis demonstrated that LiTXN1 is up-regulated (approximately 10-fold) in the stationary phase promastigotes that are enriched in the infective metacyclic form and in axenic amastigotes.¹⁸ Recently, using different approaches such as a highly sensitive radiolabelled immunoprecipitation technique¹⁹ and two dimensional electrophoresis (unpublished data), we detected LiTXN1 among the parasite excreted/secreted antigens. These observations suggest that LiTXN1 may interfere with host

immune cells, playing an important role during the infective process.

In this paper we demonstrated that rLiTXN1 can activate B cells of naïve BALB/c mice. The observed effect occurred even in the absence of accessory cells. Interestingly, rLiTXN1-induced activation seems to be B-cell specific among the lymphocyte population as no significant differences were found in the CD69 expression of CD4⁺ or CD8⁺ cells after stimulus. When cultured in the presence of rLiTXN1, BALB/c spleen cells underwent proliferation, as shown by increased thymidine incorporation. This effect was enhanced when a purified resting B-cell population was used. Because we used a recombinant protein produced in a bacterial expression system, it was essential to exclude the effect of a possible LPS contamination. Thus, complementary experiments were performed in the presence of polymyxin B. No significant alteration of the responses of spleen or isolated B cells to rLiTXN1 was observed in the presence of polymyxin B, whereas it partially abrogated the LPS effect. Also, experiments performed in C3H/HeJ, a strain characterized by its hyporesponsiveness to LPS,²⁴ confirmed that contamination of rLiTXN1 with LPS, which might account for the biological effect observed, was highly unlikely. These results are direct evidence of the capacity of rLiTXN1 to induce *in vitro* B-cell proliferation by a T-cell independent mechanism. A link between B-cell expansion, circulating antibodies and virulence enhancement has been reported in models of *Leishmania* and *T. cruzi* infections. Mice depleted of B cells by continuous administration of anti-IgM, mice that were B-cell mutants, and mice lacking circulating antibodies infected with different *Leishmania* species showed enhanced resistance compared with control BALB/c mice. Moreover, susceptibility to leishmaniasis is recovered when the B-cell population is reconstituted.^{25,26} Recently, a *T. cruzi* trans-sialidase (TS) was shown to sensitize mice towards an increased susceptibility to infection.²⁷ Also, TS-transgenic *L. major* was highly virulent.²⁸ These effects have been suggested to be related to the TS activity as a T-cell independent B-cell mitogen.²⁹ Thus, it is reasonable to assume that LiTXN1 can interfere with the host immune system before the onset of an adaptive response, playing a role in the outcome of the infection.

The functional dichotomy between Th1 and Th2 cell subsets is crucial in inducing resistance to cutaneous leishmaniasis in mice.⁴ However, in the case of visceral leishmaniasis, a counterproductive Th2 response is not apparent in mice, although it has been described in humans.³⁰ In humans, it has been demonstrated that the capacity to mount a pro-inflammatory response, with critical roles for the Th1 cytokines IL-2 and IFN- γ , is essential for the control and resolution of the infection.^{30,31} While several studies supported the notion that the shift from IL-4 to IFN- γ would provide the key to

recovery,^{32–34} recent findings have suggested that IL-10 is the most important regulatory cytokine involved in disease progression as it promotes parasite persistence.^{35,36} All these data prompted us to study the cytokine profile induced by rLiTXN1. Our results showed that *in vitro* treatment of spleen cells with rLiTXN1 induced dose-dependent secretion of IL-10 (increasing 5-fold with 5 μ g/ml and 18 fold with 50 μ g/ml of rLiTXN1, compared with unstimulated cells). In contrast, no significant differences were observed in the levels of the other cytokines measured (IL-2, IL-4 and IFN- γ) after rLiTXN1 stimulation. IL-10 is a pleiotropic Th1 suppressive cytokine which plays a pivotal role in the down regulation of macrophage activation, preventing antigen specific T-cell stimulation, and in limiting IFN- γ production.¹⁶ Acting as an immunomodulator, IL-10 promotes the attenuation of host defence mechanisms against the pathogenic invasion. In addition to findings in *Leishmania*,^{37,38} other infection models for intracellular pathogens such as *Mycobacterium avium* and *Klebsiella pneumoniae* have shown an increase in IL-10 secretion to be a common mechanism facilitating infection. Moreover, blocking of IL-10 activity with anti-IL-10 antibodies induced resistance against infection.^{39,40} Further experiments were carried out in spleen cells from mice previously immunized with rLiTXN1. The immunization did not alter the amount of secreted IL-10 in rLiTXN1 treated mice in comparison to untreated naïve mice upon *ex vivo* rLiTXN1 stimulus, suggesting that the observed effect was attributable to a direct effect on the naïve B-cell population, rather than the formation of memory. This suggestion is supported by observations that T-cell independent antigens are poor inducers of immunological memory.⁴¹ A recent review suggests the existence of B regulatory cells (Breg).⁴² Their regulatory functions are mediated by the production of the anti-inflammatory cytokine IL-10 or TGF- β , as shown in a wide range of chronic inflammation models. Also, alterations of B-cell cytokine production were reported in various parasitic infections. In *Schistosoma mansoni*, two parasite-derived antigens target B cells, triggering the secretion of IL-10, which leads to down-regulation of the Th1 inflammatory response and exacerbation of disease.^{43,44} Thus, rLiTXN1, through its capacity to stimulate IL-10, may generate an inappropriate cellular response that will contribute to the pathogenesis.

Mice and humans have phenotypically distinct populations of B cells, termed B-1 and B-2, which differ with respect to lineage origins, phenotype and anatomical origins.⁴⁵ It is well established that B-1 cells represent the main B-cell population in the peritoneal cavity of mice, while B-2 cells constitute the major fraction of the spleen B-cell population. IL-10 production is a well known feature of peritoneal CD5⁺ B1 cells.^{46,47} However, in this work, during the B-cell isolation process, the B-1 population was eliminated by depletion of CD43 expressing

B cells. This suggests that B-2 cells are direct targets for rLiTXN1 and are responsible for the IL-10 secretion. Moreover, several studies have indicated that the phenotype of IL-10 producing Breg is similar to that of B-2 but not B-1 cells.⁴² However, we cannot discount the possibility of an effect on the B-1 population, as T-cell-independent immune responses generally involve these cells.⁴⁸

Leishmania spp. uses mechanisms common to other intracellular pathogens to escape the host immune system. It has been reported that *Leishmania* possesses a large number of molecules characterized by their immunosuppressive and mitogenic like activities.^{49,50} During infection, hyporesponsiveness and polyclonal activation lead to suppressed parasite specific humoral and cellular responses, which help the parasite to establish itself in the host. The secreted/excreted proteins are among the parasitic molecules that play a major role during the establishment of infection and later in its maintenance.⁵¹ Indeed, soluble parasite derived antigens from *L. major* and *Leishmania donovani* were reported to induce polyclonal activation with the production of autoantibody activity.¹⁶ Also, an excreted factor derived from the culture medium of *L. major* was found to suppress ConA induced polyclonal activation of mouse T cells.⁵² Similar findings were reported in *T. cruzi*, where excreted/secreted antigens were found to induce high levels of non-specific antibodies, such as *T. cruzi* transialidase and the flagellar Ca²⁺-binding protein (Tc24).^{29,53} These findings contrast with those of a recent report, in which we described a secreted *Leishmania* cytosolic nicotinamide adenine dinucleotide-dependent deacetylase, *Leishmania* major silence information regulatory protein 2 (*LmSIR2*), that induced high levels of SIR2 specific antibodies. These antibodies were able to promote complement mediated killing of promastigotes and amastigotes and to inhibit their multiplication inside macrophages. Indeed, SIR2 specific antibodies, which during natural and experimental leishmaniasis are only detected in the chronic phase,^{19,54} were suggested to play a role in the early protective immune responses induced by immunization. Here, we showed that *in vivo* administration of rLiTXN1 without adjuvant induces a strong humoral response, predominantly involving the IgM, IgG1 and IgG3 isotypes, which is a typical characteristic of T-cell independent antigens.⁵⁵ Moreover, the antibodies produced reacted specifically against rLiTXN1 and not against other heterologous antigens, including DNA, Myo, BSA and other excreted/secreted *Leishmania* antigens such as LicTXNPx.⁵⁶ Interestingly, during natural human and canine leishmaniasis and experimental murine leishmaniasis, rLiTXN1 specific antibodies were present at low levels or absent.⁵⁷ Several lines of evidence have suggested that excreted/secreted proteins may play a role in the initial steps of infection. These proteins have been selected by evolution to be immunologically 'silent', allowing them to perform func-

tions that are vital for the success of parasite infection,^{58,59} However, in contrast to findings with *LmSIR2*, no anti-parasitic activity was found with LiTXN1-specific antibodies (data not shown). Further, preliminary BALB/c immunization experiments were conducted with rLiTXN1 protein. However, no significant difference in the parasite load was observed in the liver or the spleen of rLiTXN1 immunized mice compared with non-immunized mice at 6 weeks postinfection (data not shown). Nevertheless, in a recent study a trypanothione peroxidase, which is present in the same hydroperoxide cascade as trypanothione, was shown to induce long lasting protection against murine leishmaniasis when heterologous prime boost immunization was used, but not when it was delivered alone in DNA.⁶⁰ This suggests that a different vaccination approach should be investigated for rLiTXN1 to improve efficacy.

As an obligate intracellular parasite, *Leishmania* has evolved complex strategies for evading host defence mechanisms before (complement mediated killing), during (oxygen reactive species), and after (lysosomal hydrolases or nitric reactive species) entry into host cells. Overall, our present results show that LiTXN1 is a potent modulator of B-cell activity, inducing IL-10 release. We propose that LiTXN1 can play a dual role in protecting the parasite against the host immune system during the infection. In the early phase it induces immunosuppressive IL-10 secretion, which can facilitate the establishment of infection. In the later phase it may be involved in the ROS and RNS elimination cascade, facilitating maintenance of the parasite.

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Review Article

Immune Response Regulation by *Leishmania* Secreted and Nonsecreted Antigens

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Leishmania infection consists in two sequential events, the host cell colonization followed by the proliferation/dissemination of the parasite. In this review, we discuss the importance of two distinct sets of molecules, the secreted and/or surface and the nonsecreted antigens. The importance of the immune response against secreted and surface antigens is noted in the establishment of the infection and we dissect the contribution of the nonsecreted antigens in the immunopathology associated with leishmaniasis, showing the importance of these panantigens during the course of the infection. As a further example of proteins belonging to these two different groups, we include several laboratorial observations on *Leishmania* Sir2 and LicTXNPx as excreted/secreted proteins and LmS3arp and LimTXNPx as nonsecreted/panantigens. The role of these two groups of antigens in the immune response observed during the infection is discussed.

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1. INTRODUCTION

Leishmaniasis are parasitic diseases, caused by protozoan parasites of the *Leishmania* genus, associated with significant morbidity and mortality in tropical and subtropical regions and in the Mediterranean basin. The disease has a wide range of clinical manifestations that depend not only on the infecting *Leishmania* species but also on the immune status of the host [1]. The most extensively studied leishmanial disease is the cutaneous form caused by *L. major* or *L. tropica* in the old world and *L. braziliensis* in the new world. It usually appears as a skin ulcer or dermal granuloma, which may take up to several months or years to heal [2]. With *L. braziliensis*, the infection may also spread to other cutaneous sites, like mucosal membranes giving origin to the mucocutaneous form of the disease. The most serious form of the disease is the visceral one that, if untreated, gives rise to a high mortality rate. It is characterized by fever, cachexia, hepatosplenomegaly, and hypergamaglobulinemia and is caused by members of the *L. donovani* complex (*L. donovani* in the old world, *L. infantum* in the Mediterranean basin and *L. infantum chagasi* in the New World) [3].

Leishmania is a digenetic protozoan that is transmitted to the mammalian host by sandflies of the genus *Phlebotomus*

in the old world and *Lutzomyia* in the new world. In the alimentary tract of the insect vector, the parasite exists extracellularly as a flagellated motile form, the promastigote. During the insect blood meal, the infectious developmental form, metacyclic promastigotes, is injected into the dermis and phagocytized by resident macrophages within which the parasite differentiates into the nonmotile amastigote form and multiplies. Moreover, other cells such as fibroblasts and dendritic cells may also harbour parasites [4]. The cycle is completed when the sandfly takes another blood meal recovering free amastigotes or infected macrophages.

During an infection, the parasites have a remarkable adaptative capacity as they are able to survive inside phagocytic cells. These cells are responsible for the microbicidal and antigen-presenting functions however they serve as a safe habitat for the parasite. The existence of inbred mice, which are either susceptible (Balb/c) or resistant to infection (C57BL/6, CBA, C3H.HeJ) has helped to elucidate the protective or nonprotective role of cytokine and T-helper cell subsets and also the role of different leishmanial antigens in the immune evasion mechanism. Thus, it became generally accepted that resistance against leishmaniasis is associated with the production of IL-12 by antigen presenting cells (APC) macrophages and dendritic cells, leading to the

differentiation and proliferation of the Th1-subset of CD4⁺ T-cells producers of IFN- γ . This will ultimately lead to the activation of parasite-infected macrophages that, through the induction of effector molecules as nitrogen and oxygen reactive species, will kill the intracellular parasites [5]. In contrast, failure to control the infection has been associated with the production of anti-inflammatory cytokines as IL-4, IL-10, IL-13 and TGF- β [6]. Given the ancient evolutionary divergence in *Leishmania* species, it is not surprising that the control of the different *Leishmania* driven diseases is related to different immunological properties. Hence, while in cutaneous leishmaniasis, IL-4 has been implicated in disease progression, in visceral leishmaniasis its importance has been ruled out [7]. In the latter, IL-10 has been shown to be the major immunosuppressive cytokine along with TGF- β . Overall, it suggests that it is the overshadowing of the Th2 response by a Th1 cell associated response that leads to the control of the infection [8]. Moreover, the real contribution of the humoral response is still under debate, however studies in different intracellular pathogens have shown that antibodies can also have a function in restricting the infection when the parasite is exposed to the extracellular milieu [9]. Consequently, in leishmaniasis, the induction of specific humoral responses to parasite antigens would, theoretically, be able to neutralize the parasite whether as free promastigotes, after the inoculum, or as amastigotes, when released from the infected macrophages, contributing to develop a protective response [10]. However, until now, no effective vaccine against human leishmaniasis is available for clinical use [3].

Leishmania parasites inside their hosts do not behave inertly. Rather, the virulence related to their pathology seems to be linked to an induced lack of immune response control. The parasite actively secretes proteases and other molecules that affect host immune system (cells and cytokines) facilitating the infection process. In addition, the parasite possesses intracellular nonsecreted antigens, members of conserved protein families, which are believed to contribute to the chronic immunopathology, observed in leishmaniasis. Here, we review these two groups of relevant parasite molecules, illustrated with laboratory observations of proteins belonging to the secreted and nonsecreted groups of antigens. Finally, we discuss their differential role in *Leishmania* infection and persistence as well in the development of a protective immune response.

1.1. The importance of the secreted versus nonsecreted antigens

Leishmania virulence has been explained using two different groups of parasite molecules, the secreted and surface and the intracellular molecules [11]. This model proposes that the secreted and surface molecules will be mostly important for the establishment of infection, protecting the parasite from the early action of the host immune system, acting as invasive/evasive determinants. According to this model, the intracellular molecules will be ultimately responsible for the disease phenotype [11].

1.2. Surface and secreted molecules

The secreted proteins have distinct functions during *Leishmania* infection. First, they play a role in the establishment of the infection [12] in conjunction with important elements existent in the saliva of the sandfly vector [13, 14]. In a second phase, they contribute to the maintenance of the infection by interfering with the macrophagic microbicidal functions, cytokine production, antigen presentation, and effector cells activation. This is achieved by repression of gene expression, post-translation protein modification or degradation, and by activation of suppressive pathways and molecules [15]. This macrophagic anergy enables the continuous multiplication of the amastigote form. The bulk of the knowledge on surface and secreted molecules of *Leishmania* is focused on lipophosphoglycan (LPG), on the promastigote surface protease named glycoprotein 63 (gp63), glycosylinositol phospholipids (GIPLs), cysteine peptidases and on a few others like β -mercaptoethanol activated proteases, acid phosphatases and chitinases. The importance of some of these molecules in the establishment of the infection is well documented [15, 16], but the real contribution of the secreted molecules remains elusive due to the difficulty of the intramacrophagic studies.

After entrance into a susceptible mammalian host, the *Leishmania* promastigotes are targeted by the host immune system. Serum components, like the complement system represent the first challenge following entrance into the bloodstream. Procyclic promastigotes are highly susceptible to complement action, unlike the metacyclic that can avoid complement mediated lysis [17]. This remarkable difference is mostly due to the surface molecule in *Leishmania*, the LPG. Composed mainly of repetitive units of a disaccharide and a phosphate, LPG is linked to the membrane by a glycosylphosphatidylinositol anchor [18]. The LPG is longer in metacyclic promastigotes preventing the attachment of C5b-C9 subunits of the complement complex avoiding its lytic action [17]. The relevance of LPG is not limited to complement resistance. Its importance is stated by several studies using either purified LPG or mutant strains. The LPG is implicated in several processes including the binding to the epithelial cells of the sandfly midgut [19], receptor mediated phagocytosis of macrophages through the CR3/CR1 ligand or the manose-fucose receptor (in conjunction with gp63) [20, 21], toll-like receptor 2 signalling [22], stimulation of NK cells [23], inhibition of phagosome-endosome fusion [24–26], and inhibition of phagosome-derived superoxide [27]. Several attempts to use LPG to confer protection were unproductive [28, 29]. Constitutively shed by several *Leishmania* species, the LPG is the paradigm molecule referred to as evasive and invasive. After the initial steps of infection, LPG is downregulated being almost absent from amastigotes [30].

Another molecule implicated in the invasive and evasive mechanisms is gp63. This protein is the most abundant in the parasite surface, although 10 fold less abundant than LPG [30]. In the promastigote form, gp63 is in the surface of the parasite under the LPG coat and is involved

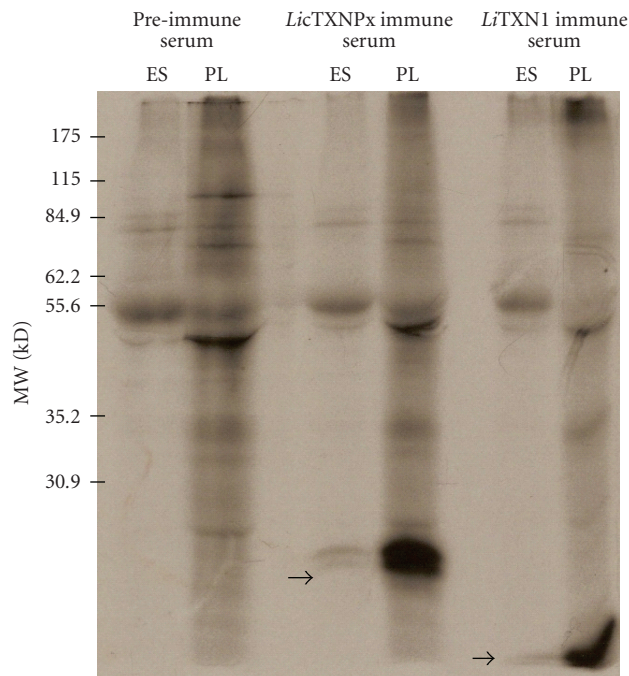


FIGURE 1: The *LicTXNPx* and *LiTXN1* are excreted/secreted proteins. Autoradiography of [^{35}S] methionine labelled *L. infantum* promastigotes lysate (PL) and excreted/secreted antigens (ES), after 3 hours of incubation experiments, immunoprecipitated in the presence of immune anti-*LicTXNPx* or anti-*LiTXN1* sera or with a preimmune serum.

in *L. donovani* promastigote multiplication [31]. Like LPG, gp63 was shown to be implicated in complement resistance, in *L. major* and *L. amazonensis*, by mediating the interconversion of C3b to C3bi [32]. This interconversion favours the internalization via CR3 avoiding the oxidative burst. The binding of gp63 to fibronectin receptors favours the parasite uptake into the macrophage [33]. Furthermore, gp63 is an endopeptidase with the potential to degrade immunoglobulins, complement factors, and lysosomal proteins [34]. The optimal proteolytic activity of gp63 is at pH 4 that may indicate some active proteolytic function in the amastigote stage [34, 35]. Despite this, gp63 expression is downregulated in amastigotes [36]. In spite of being a virulence factor in most *Leishmania* species, immunization trials with gp63 were unable to protect mice from infectious challenge [37]. Moreover, gp63 mutation in *L. major* did not impair in vitro intramacrophagic survival [38]. So the importance of gp63 in the course of the infection remains elusive. The GIPLs are molecules 10 times more abundant than LPG on the parasite surface, although like gp63 they are physically under the LPG coat [39]. The GIPLs were described in *L. major* as having a protective role at the parasite surface by modulating the expression of nitric oxide synthetase in murine macrophages [40, 41]. Another interesting group of proteins are the cysteine proteases. In *L. mexicana*, this family of proteins seems to be associated with disease progression [42]. Cysteine protease activity can be found at the parasite surface or inside

the macrophage endoplasmatic reticulum, probably associated with proteases released in the phagolysosome by *Leishmania*. The inhibition of major histocompatibility complex class II molecules in macrophages seems to involve, in *L. amazonensis*, the direct sequestering of these molecules following cysteine-peptidase-dependent degradation [43, 44]. Also, cysteine peptidase activity was demonstrated in *L. mexicana* to induce IL-12 repression and degradation of NF-kB [45]. It is still worthy to mention some other secreted proteins described as virulence factors, like the *L. mexicana* chitinase [46] and the *L. donovani* acid phosphatases [47–50]. An in depth study of the *Leishmania* secretome is missing. The most remarkable effort was done by Chenik and colleagues that were able to screen 33 different proteins using an *L. major* cDNA library and a rabbit immune sera raised against the secreted proteins [51]. Nine of them were already described as excreted/secreted proteins in *Leishmania* or other species, 11 corresponded to known proteins but not characterized as secreted and the other 13 were completely new and uncharacterized proteins [51]. This shows how little is known about the *Leishmania* secretome since only a few proteins are extensively characterized [52–56]. It is already known that total *L. major* secreted molecules, described as highly immunogenic [54, 57–59], can confer protection from infectious challenge [57, 59]. So it is obvious that somewhere among the *Leishmania* secreted proteins exist future candidates for vaccine design and drug targets. Nonetheless, one of the problems in vaccine design using surface or secreted/excreted proteins is the fact that these proteins are naturally exposed to the immune system. Chang et al suggest that these secreted/excreted proteins were evolutionarily selected becoming immunologically “silent” [60]. This fact implies that secreted proteins that have a specific function in the establishment of the infection will be “silent,” allowing them to perform their vital functions unchecked by the host immune system [11, 12]. This will be more significant for the proteins involved in the first steps of infection, while the parasite is still exposed to the extracellular environment. As an example of this fact, we present three distinct proteins: a cytosolic trypanothione peroxidase of *L. infantum* (*LicTXNPx*) [61], the *Leishmania* silent information regulator 2 (*Sir2*) [52], and a trypanothione of *L. infantum* (*LiTXN1*) [62]. All are *Leishmania* secreted proteins (Figure 1) [52], that show distinct immunological properties. A high antibody titre against the *LicTXNPx* was detected in children [63]. This antibody titre is maintained during the *Leishmania* infection and decreases after its resolution [63]. Despite its high immunogenicity when tested in vitro or in vivo using the Balb/c model, this excreted/secreted protein did not show immunomodulatory properties (Figures 4, 5, and Table 1) and provided no protection against the infectious challenge (data not shown). On the other hand, the *Leishmania* *Sir2* is a typical poorly immunogenic secreted antigen (Figure 2) characterized as a virulence factor [64]. Infectious challenge after *Leishmania* *Sir2* immunization results in a decreased infectivity in the acute phase (Figure 3). This could be partially due to the production of lytic and neutralizing antibodies [65]. The immunization leads to a significant decrease of the spleen

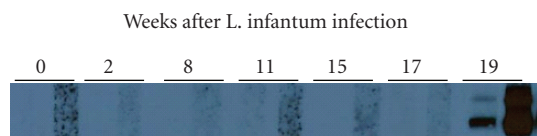


FIGURE 2: Antibodies against *Leishmania* SIR2 protein in the sera of chronically *L. infantum* infected Balb/c mice. Sera from 10^8 intraperitoneal (i.p.) *L. infantum* promastigotes infected Balb/c mice after 2, 8, 11, 15, 17, and 19 weeks, were used in a western blot against $1\ \mu\text{g}$ of rLiSIR2 at two different dilutions, 1 : 200 and 1 : 50 (left and right lanes, respectively, for each different serum). A 0 weeks serum was obtained from noninfected mice.

and liver parasite load at two weeks post infection (Figure 3) [65]. However, it is incapable by itself of resolving the infection, as seen six weeks after infection, where there is no significant difference between the immunized infected group and the infected control group (Figure 3). Certain secreted proteins seem to function as immunomodulatory components, acting as host immune evasive proteins. As an example, another excreted/secreted *Leishmania* protein, LiTXN1 (Figure 1), is capable to increase IL-10 splenocyte secretion (Table 1), a major immunosuppressive cytokine (manuscript in preparation). LiTXN1 can be among the proteins responsible for a transient immunosuppressive state that can favour the parasite internalization and colonization of the host cells. These examples show that among the secreted proteins we can find proteins naturally immunogenic, albeit nonprotective, like LiTXNPx while others less immunogenic show interesting properties in terms of protection probably due to the disruption of their in vivo functions, *Leishmania* Sir2, or by their immunomodulatory properties, LiTXN1. Unfortunately, the reduced immunogenicity of the most interesting secreted proteins probably will prevent their identification by serological based approaches [51].

The reduction of the secreted/excreted proteins to the given examples is an oversimplification. However, it is obvious that much more work is needed in this area, especially in the huge black hole of knowledge that concerns the interaction between host cell and *Leishmania* at a molecular level. Since most of the studies have been done using infection-phenotype approaches, little is known about the true agents involved in macrophagic disruption [16, 58, 68, 69]. We suggest that amastigote secreted proteins will be more immunogenic and can have interesting immunomodulatory properties since they have not been under the selective pressure as the promastigote secreted proteins. The selective pressure of the host immune system is a powerful driving force in evolution, as demonstrated in the case of *Schistosoma mansoni* that has the ability to completely evade the host immune system rendering itself “invisible” [70].

1.3. Panantigens—nonsecreted proteins

Human visceral leishmaniasis, unlike cutaneous leishmaniasis is characterized by high anti-*Leishmania* antibody titres [71, 72]. The role of these antibodies is still unclear as there

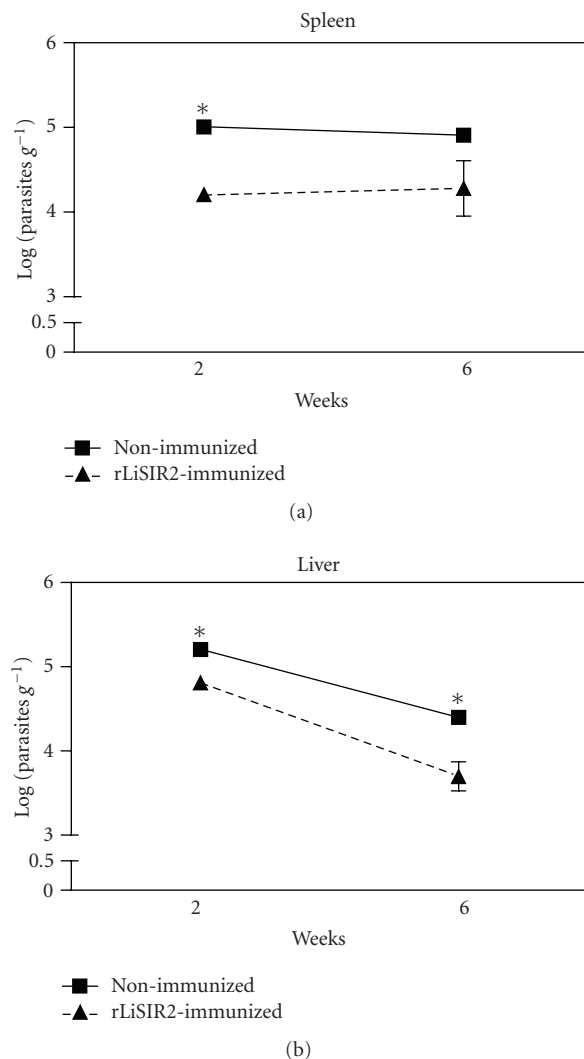


FIGURE 3: The recombinant *Leishmania* SIR2 immunization reduces the parasite load in an acute phase of *L. infantum* Balb/c mice infection. The immunized mice ($\blacktriangle$) received 3 i.p. injections of recombinant *Leishmania* SIR2 ($50\ \mu\text{g}$) once a week and infected 2 weeks after the last immunization with 10^8 *L. infantum* stationary phase promastigotes. The nonimmunized mice ($\blacksquare$) were subjected to the same protocol but received PBS instead of recombinant *Leishmania* SIR2. The mice were sacrificed after 2 and 6 weeks of infection and the parasite load in the spleen and liver determined by the organ limiting dilution method [66]. The data represent means and standard deviations for three mice and are representative of two independent experiments. Statistical analysis was performed using Student *t*-test. Statistically, significant differences between immunized and nonimmunized mice are indicated. * $P < .05$.

seems to be no relation with the progression or resolution of the infection [58, 73, 74]. This exuberant humoral response against promastigote and amastigote antigens (fractions or total protein extract or specific *Leishmania* proteins) has been exploited for serodiagnosis with different degrees of success [58, 63, 74, 75]. Interestingly, one of the most sensitive techniques using recombinant *Leishmania* proteins does not involve surface molecules like LPG or gp63 but

TABLE 1: Immunomodulatory properties of several *Leishmania* proteins

Protein	Properties	References
<i>Leishmania</i> Sir2	Secreted, B-cell activator, induces lytic, and neutralizing antibodies	[64, 65]
LicTXNPx	Secreted, elicits strong humoral response and has no influence on cytokine production	[63]
LimTXNPx	Nonsecreted, decreases IL-4 secretion both in vitro and in vivo	Figure 3
LrTXN1	Secreted, poorly immunogenic, induces IL-10 secretion both in vitro and in vivo	(Manuscript in preparation)
LmS3arp	Nonsecreted, B-cell polyclonal activator, inhibits T-cell proliferation, and downregulate IL-2, 12 and IFN- γ in splenocytes	[67]

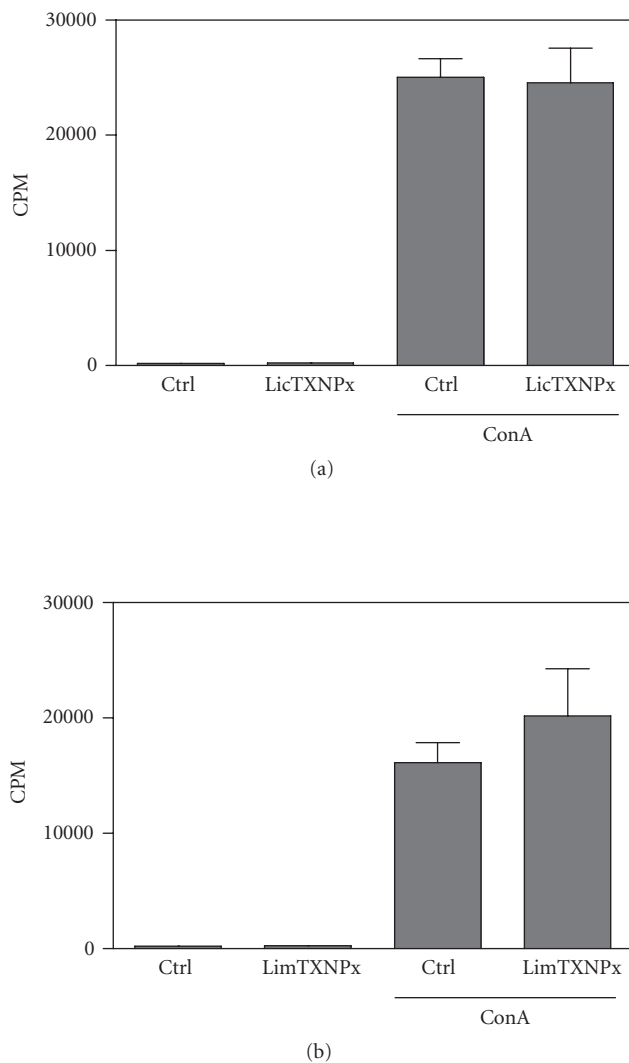


FIGURE 4: No effect of *rLicTXNPx* and *rLimTXNPx* (a) on spleen cell proliferation. Spleen cells from normal Balb/c mice were cultured for 48 hours (2.5×10^5 cells/well) in the presence or absence of concanavalin A (ConA) ($5 \mu\text{g/ml}$) with or without *rLicTXNPx* and *rLimTXNPx* (b) ($10 \mu\text{g/ml}$). The cells were pulsed with [methyl- ^3H] thymidine in the last 8 hours of culture, and cpm (scintillations per minute) were determined. The data represent mean cpm and standard deviations from triplicate cultures of spleen cells from three mice analyzed individually. One of three independent experiments is depicted.

intracellular proteins like histones [75]. The screening of *Leishmania* expression libraries or total protein extract with serum from infected patients has unveiled several major immunogens [76–79]. Among these immunogens, nonsecreted proteins like heat shock proteins, ribosomal proteins and histones were described [76, 77, 80]. These highly-conserved proteins that elicit strong immune responses are generally designated as panantigens [81]. The elevated antibody titre against conserved proteins can be the direct result of B-lymphocytes polyclonal activation similar to what is found in Chagas disease [82, 83] or in autoimmune diseases [84]. Furthermore, in the Balb/c mouse model, an *L. major* protein homologue to the mammalian ribosomal protein S3a, *LmS3arp*, (Table 1) is able to elicit an unspecific activation of B-lymphocytes with the production of autoreactive antibodies [67]. Despite this, in natural infections, the humoral and cellular responses are highly specific with no significant autoantibody production [80, 81, 85]. Moreover, the epitope mapping of several *Leishmania* panantigens tends to reveal *Leishmania* unique epitopes that elicit strong immune responses [79–81, 86, 87]. There is practically no response to the homologous regions in these proteins, which argues against the nonspecific polyclonal activation as the source of reactivity against *Leishmania* panantigens [11, 81]. So, it is expected that these proteins are presented to the immune system during the natural course of the infection. Unlike secreted and surface proteins that are exposed and can be processed by the host immune system, the intracellular proteins are not. One must expect that the contact between the immune system and these proteins happens only upon the parasite destruction. Subsequently, one obvious source of intracellular proteins is the parasites from the initial inoculum some of which are destroyed. Furthermore, it was recently demonstrated that the presence of apoptotic parasites in the initial inoculum is a requisite for disease development [88]. Albeit the small number of parasites in the initial inoculum is not sufficient to explain the physical expansion of cell populations and immune mediators during the course of infection, it is a fact that panantigens are exposed long before the onset of any visible symptoms [88]. This initial release of panantigens may function in conjugation with the secreted and surface proteins acting as a transient “smoke screen” that enables the onset of the initial infection by viable parasites. The immune response developed against the panantigens may contribute to hide the parasite molecules

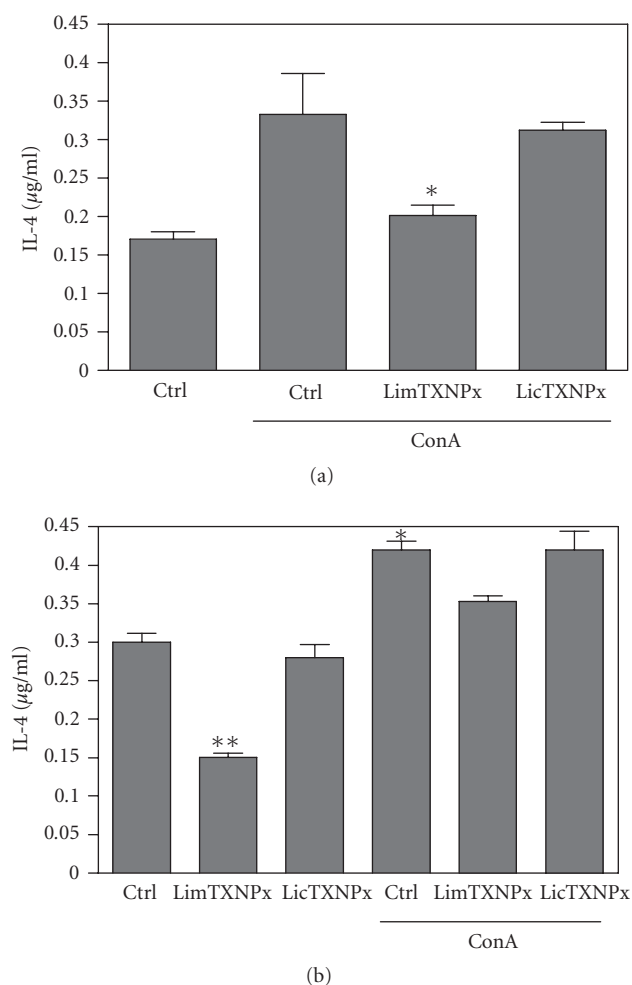


FIGURE 5: Levels of IL-4 in the supernatants of spleen cells from rLicTXNPx or rLimTXNPx treated and untreated Balb/c mice. The spleen cells from untreated (a) and treated (b) Balb/c mice (50 µg of rLicTXNPx or rLimTXNPx i.p. injected once a week for 3 weeks followed by 2 weeks before the spleen cells were recovered) were incubated with rLicTXNPx or rLimTXNPx (10 µg/ml) in the presence or absence of ConA (50 µg/ml) for 48 hours. The levels of IL-4 were determined by ELISA in comparison with a standard curve using the recombinant IL-4. The data represent means and standard deviations for triplicate cultures of spleen cells from three mice. The results are from a representative experiment of three carried out independently. Statistical analysis was performed using Student *t*-test. Statistically, significant differences is indicated. **P* < .05 and ***P* < .01.

involved in the invasion of the phagocytic cells. Moreover, the humoral profile suggests a steady release of panantigens during the infection [58, 73, 74]. It is also [81] suggested that panantigens originate from the residing parasite population either by the destruction of intracellular amastigotes by active macrophages or by the destruction of amastigotes that burst from macrophages or even by the spontaneous cytolysis of amastigotes inside the infected cells [11]. In active leishmaniasis, there seems to be a general anergy in infected macrophages that leads to impaired functioning

[16, 89–92]. So, in this case, it is not expected that panantigens may result from the macrophage mediated elimination of *Leishmania*, as it will lead to the resolution of the infection. Although free amastigotes can infect macrophages directly, they are almost undetectable even in heavily infected hosts. Thus, their contribution to the pool of panantigens should be diminished [11]. The low speed of intracellular amastigotes multiplication and their capacity to delay apoptosis in heavily infected phagocytes [60] enables a lasting coexistence in infected macrophages. The most viable theory for the phased release of panantigens would be the spontaneous cytolysis (described as apoptosis by some authors) of intracellular amastigotes [93]. The effect of the panantigen release is gradual and more significant as the infection develops and the parasite burden augments explaining the increasing intense immunopathology associated with *Leishmania* infection [11]. This increase in panantigen release can be extrapolated in correlation with panantigen antibody titres and parasite burden as seen for the *Leishmania* kinesin like protein, k39 [81, 94]. Another protein that shows similar characteristics to k39 is the LicTXNPx which has also the ability to induce a high quantity of nonprotective antibodies both in natural or experimentally infected dogs (unpublished data) and in infected humans [63]. This induction can be done by direct activation on B-cell populations with clonal expansion as described for *Leishmania* Sir2 [65], which seems not to be the case since little or no antibodies for LicTXNPx are seen in HIV patients with leishmaniasis (unpublished data), as was observed for k39 [95]. This suggests the existence of specific T-cell epitopes in LicTXNPx. The nature of these epitopes will not be similar to those of k39, because the latter contain repetitive motifs that will contribute significantly to the clonal expansion of B-cells. For LicTXNPx, the strong immune response observed should be due to the formation of highly stable multimeric structures characteristic of this protein [96]. The nonprotective antibody titres induced by LicTXNPx seem to be transient and associated only with the immunopathology as they disappear after a period of time, unlike other *Leishmania* specific antibodies simultaneously in circulation [63]. These antibodies may contribute to the impairment of bone marrow and spleen [11].

The capacity of panantigens to modulate the immune system can be related to the fact that these intracellular proteins were not selected by the immune pressure, unlike the secreted and surface proteins. Hence, in the right conditions, they can provide the immunomodulatory properties needed for vaccine design. The most prominent intracellular proteins used in vaccine design are still LACK and LmSTI1 that are able to induce protective responses with a parasite-specific Th1 immune response (high IFN-γ but not IL-4 secretion) [87, 97]. Among the *Leishmania* proteins studied by our group, a mitochondrial trypanothione peroxidase (LimTXNPx; Table 1), homologous to LicTXNPx, is able to induce down regulation of IL-4, a Th2 cytokine, in splenocytes both in vitro and in vivo (Figure 5) though unable to induce significant protection (data not shown). It is noteworthy that similar proteins such as LicTXNPx and LimTXNPx are able to elicit distinct immune responses.

LicTXNPx is secreted inducing only the production of non-protective antibodies, while its related intracellular counterpart *LimTXNPx* has immunomodulatory properties interfering with cytokine production (Figure 5). This can be a good example of the type of evolutionary pressure induced by the immune system, in which two related proteins have distinct immunomodulatory properties (Figures 4, 5). It suggests that the host immune system selects characteristics in the exposed proteins that are either innocuous or nondeleterious to the parasite. Since this does not occur in the intracellular proteins they can retain distinct immunoregulatory properties that could be useful in vaccine design.

2. CONCLUDING REMARKS

Taken altogether, these observations support the idea that secreted and surface proteins tend to be poor or nonprotective immune modulators, like *LicTXNPx*. Nonetheless, their use in vaccine could induce short-lived protection probably due to the disruption of their biological activity or by production of lytic antibodies, as seen with *Leishmania* Sir2. Intracellular components like *LmS3arp* and *LimTXNPx* tend to have defined immunomodulatory properties. *LmS3arp* is able to induce polyclonal activation of B lymphocytes while *LiLimTXNPxTXNPx* confers a nonprotective downregulation of IL-4 secretion by splenocytes.

Using the basic knowledge acquired in the study of the immune response against *Leishmania* in different murine models, one can look for proteins that induce the immunological phenotype needed for protection. Therefore, our data suggests that in vaccine development, the conjugation of secreted and surface proteins with intracellular components should provide a more efficient protection. Hence, the impairment of the parasite entrance in the host cells, either by lytic antibodies or by the disruption of protein function, will delay the onset of the immune suppression associated with *Leishmania*. The parasite elimination could be achieved through a protective cellular response, induced by the intracellular parasite components present in the vaccine.

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***Leishmania* cytosolic silent information regulatory protein 2 deacetylase induces murine B-cell differentiation and *in vivo* production of specific antibodies**

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Summary

In previous studies, we identified a gene product belonging to the silent information regulatory 2 protein (SIR2) family. This protein is expressed by all *Leishmania* species so far examined (*L. major*, *L. infantum*, *L. amazonensis*, *L. mexicana*) and found to be crucial for parasite survival and virulence. In the present study, we investigated whether a *Leishmania* SIR2 recombinant protein (LmSIR2) would affect T- and B-cell functions in a murine model. *In vitro* treatment of spleen cells from normal BALB/c mice with LmSIR2 showed increased expression of CD69 on B cells. This effect was not abolished by the addition of polymyxin B. Intravenous injection of LmSIR2 into BALB/c mice induced increased spleen B cell number by a factor of about ≈ 1.6 , whereas no modification occurred at the level of CD4⁺ and CD8⁺ cells. Furthermore, intraperitoneal injection of LmSIR2 alone without adjuvant into BALB/c mice or nude mice triggered the production of elevated levels of LmSIR2-specific antibodies. The analysis of specific isotype profiles showed a predominance of immunoglobulin G1 (IgG1) and IgG2a antibody responses in BALB/c mice, and IgM in nude mice. Moreover, the anti-LmSIR2 mouse antibodies in the presence of complement induced the *in vitro* lysis of *L. infantum* amastigotes. In the absence of complement, the antibodies induced significant inhibition of amastigotes development inside macrophages. Together, the current study provides the first evidence that a *Leishmania* protein belonging to the SIR2 family may play a role in the regulation of immune response through its capacity to trigger B-cell effector function.

Keywords: *Leishmania*; SIR2; B cells; specific antibodies; leishmaniasis

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Introduction

Protozoans of the genus *Leishmania* are obligate intracellular parasites causing a wide spectrum of human and veterinary diseases known as leishmaniasis. The disease causes a broad range of clinical symptoms, from a self-healing localized cutaneous form to the disseminated potential lethal infection, depending on the complex interaction between the infecting *Leishmania* species and the host immune response.¹ Active visceral leishmaniasis (VL), caused by members of *Leishmania donovani*

complex (*L. donovani* in the Old World, *L. infantum* in the South-east of Europe and Mediterranean area and *L. chagasi* in the New World) is a progressive infection characterized by fever, cachexia, hepatosplenomegaly and hypergammaglobulinaemia, symptoms that develop in both humans and dogs.^{2,3} The interaction between the parasite and its host led to a variety of disturbances in the immune system. One of the immunological hallmarks of VL is a profound suppression of parasite-specific T-cell mediated immune responses⁴ associated with a remarkable increase in the serum immunoglobulin levels as a

Abbreviations: VL, visceral leishmaniasis; CML, complement-mediated lysis; FCS, fetal calf serum; LPS, lipopolysaccharide; mAb, monoclonal antibody; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; i.p., intraperitoneally; i.v., intravenously; MFI, mean fluorescence intensity; ELISA, enzyme-linked immunosorbent assays; RLU, relative light units; WT, wild type; FACS, fluorescence-activated cell sorter.

result of polyclonal B-cell activation, which invariably produce large amounts of parasite non-specific antibodies with self autoreactivity particularly the immunoglobulin M (IgM) and IgG isotypes.⁵ Using a mechanism common to other intracellular pathogens, *Leishmania* spp. displays mitogenic-like activities that produce a non-specific general activation of lymphocytes. Thus, previous reports have characterized immunosuppressor factors and mitogenic molecules within the parasite antigens.^{6,7} Thus, it seems that the persistence of infection could be related somehow to the immunosuppression phenomenon, the non-specific responses, and the development of auto-immune processes in relation to defined parasite molecules. Therefore, it seems reasonable to assume that induction of specific responses against 'immuno-dominant', 'immunopathological' or 'protective' antigens, which could interfere with some of the biological activities, might be among the most effective means to block the development of pathogenic processes.⁸

It is generally accepted that protective immunity against leishmaniasis is associated with a classical cell-mediated immune response, rather than a humoral one. This view has been interpreted in context of the T helper 1 (Th1)/Th2-biased responses in the mouse model of *L. major*. However, in visceral leishmaniasis, while a interleukin-12 (IL-12)-driven type 1 response is imperative for protection, type 2 responses (that support humoral immunity) also play a role, although minor, in promoting resistance.⁹ Although the real contribution of antibodies is still under debate, studies in different intracellular pathogens have shown that antibodies can also have a function in restricting the infection when the parasite is exposed to the extracellular milieu.¹⁰ In leishmaniasis, interaction of antibodies with the parasite could occur when the promastigote infects an individual or when the amastigotes are released from the infected macrophages.¹¹ Moreover, their possible role in protection was shown by passive *in vivo* transfer of monoclonal antibodies.¹²

In previous reports we have characterized silent information regulatory 2 protein (SIR2) proteins from *L. major* (LmSIR2)¹³ and *L. infantum* (LiSIR2)¹⁴ while two other related protein sequences can be found in the *Leishmania* genome database (*L. major* sirtuin (CAB55543), *L. major* cobB (LmjF34.2140). Molecular and biological approaches allowed us to show the potential role of LmSIR2 and LiSIR2 genes in parasite survival.^{14,15} Furthermore, using LmSIR2 as a probe, immunological investigations demonstrated that LmSIR2 was highly immunogenic during natural human and canine infections.¹⁶

In the current study, we investigated whether LmSIR2 would trigger the activation of murine lymphocytes, and surprisingly murine B cells, but not T cells, respond strongly and directly to stimulation with LmSIR2. The B cells underwent differentiation *in vivo* as evidenced by the

production of significant levels of specific antibodies. Our data suggest an immunoregulatory role of leishmania SIR2 protein during leishmaniasis.

Materials and methods

Leishmania strain and mice

A cloned line of *L. infantum* (MHOM/MA/67/ITMAP-263, wild type (WT)) was used for the infectious challenge. A cloned line of *L. infantum* expressing the luciferase (*LUC*) gene derived from WT¹⁷ was used in all *in vitro* experiments. Six to seven-week-old BALB/c male mice were obtained from Harlem Iberica (Spain). Seven-week-old BALB/c athymic (nude) mice were obtained from Charles River (L'Arbresle, France).

Purification of LmSIR2

The *L. major* SIR2 was obtained as recombinant protein containing six histidine residues at its N-terminal. The construction of plasmid and purification of protein have been described elsewhere.¹⁸ The purity of the recombinant protein (LmSIR2) was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie blue. For biological assays, the protein was dialysed against phosphate-buffered saline (PBS) in decreasing concentrations of urea. The final dialysis was performed against PBS. The protein concentration was determined using the Folin procedure.¹⁹ To eliminate endotoxins, the recombinant protein was passed through an EndoTrap®red column (Profos, Germany) following manufacturer's instructions.

Mouse injections

In the case of BALB/c mouse experiments two different routes of administration were followed. One group of BALB/c mice was injected three times intraperitoneally (i.p.) at a 7-day interval with 50 µg of LmSIR2 in 300 µl of PBS, or 300 µl of PBS (control group). Two weeks after final immunization, spleens and sera were collected. Another group of BALB/c mice received one intravenous (i.v.) injection of 20 µg of LmSIR2 in 150 µl of PBS, or 150 µl of PBS (control group). After 72 hr, mice were killed and spleens collected. In the case of BALB.nude mice, two groups of five mice each were injected three times i.p. at a 7-day interval with either 50 µg of endotoxin-free LmSIR2 in 400 µl of PBS, or 400 µl of PBS (control group).

BALB/c mice immunization and infection

For immunization, one group of BALB/c mice was injected three times i.p. at a 7-day interval with 50 µg of

LmSIR2 in 200 µl of sterile PBS. A control group was injected with 200 µl of sterile PBS in a similar schedule. Fifteen days after the last immunization both groups were challenged with 10^8 stationary-phase WT promastigotes resuspended in 200 µl of sterile PBS.

Spleen B-cell isolation

After cervical dislocation, the spleens were removed and homogenized in a Petri dish to obtain a single-cell suspension. After two washes in RPMI-1640 culture medium (Cambrex, Saint Beaulieu, France), the cells were adjusted to 10^7 /ml in RPMI-1640 culture medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 20 mM HEPES. Spleen B-cell isolation was carried out using a B Cell Isolation Kit, MACS® (Miltenyi Biotec, Auburn, CA) as described by the manufacturer. The effectiveness of B-cell purification was determined by double labelling with specific monoclonal antibodies (mAb; anti- μ^+ , anti-CD4 $^+$ and anti-CD8 $^+$) and further fluorescence-activated cell sorting (FACS) analysis. After purification, we obtained over 97% B-cell purity.

Flow cytometry determinations

Spleen cells or isolated B cells from normal or LmSIR2 immunized mice and their respective controls were prepared in order to obtain single-cell suspensions. The cells were washed by centrifugation and resuspended in PBS supplemented with 2% FCS. A total of 10^6 viable cells were incubated for 30 min at 4° with saturating concentrations of phycoerythrin (PE)-conjugated mAb to CD69 plus fluorescein isothiocyanate (FITC)-conjugated mAbs to either CD4, CD8 α or IgM (anti- μ) from BD Pharmingen (San Diego, CA). After two washing steps with PBS–2% FCS, the cells were analysed by flow cytometry in a FACS Scan equipped with CellQuest Pro software (Becton Dickinson, San Jose, CA). Lymphocytes were selected on the basis of forward scatter/side scatter values and dead cells were excluded from all samples by propidium iodide labelling.

To evaluate the binding of antibodies to LUC-recombinant WT amastigotes, 2.5×10^5 parasites were washed twice in PBS containing 0.5% of bovine serum albumin (PBS–BSA) and incubated for 30 min at 4° with mouse anti-SIR2 immune serum diluted 1 : 10 in PBS–BSA. After two washes with PBS–BSA, the parasites were incubated for 30 min at 4° with FITC-conjugated goat anti-mouse IgG diluted 1 : 5000 in PBS–BSA. Labelled amastigotes were washed twice and resuspended in 500 µl of PBS–BSA followed by flow cytometry analysis. A preimmune serum was used as control. Results were expressed as mean fluorescent intensity (MFI).

Enzyme-linked immunosorbent assays (ELISA) for immunoglobulins

Ninety-six-well flat-bottomed microtitre plates (Greiner, Laborchnik, Solingen, Germany) were coated overnight at 4° with one of the following reagents in 0.01 M carbonate/bicarbonate buffer pH 8.5: unlabelled goat anti-mouse immunoglobulin (5 µg/ml), total *L. infantum* protein extract (10 µg/ml), LmSIR2 (5 µg/ml), whale skeletal muscle type II myoglobin (MYO, 5 µg/ml), keyhole limpet haemocyanin (KLH, 5 µg/ml), LmS3a (5 µg/ml). The plates were washed with PBS containing 0.1% Tween-20 (PBS-T) and blocked with PBS 1% gelatine (200 µl/well) for 1 hr at 37°. The plates were incubated at 37° with serial dilutions (for total titres) or 1 : 100 dilutions in triplicate (for specific antibody reactivity) of each serum for 2 hr. After washing with PBS-T, the plates were incubated for 30 min at 37° with peroxidase-labelled goat anti-mouse immunoglobulin isotypes (anti-IgM, anti-IgG, anti-IgG1, anti-IgG2a) and developed with 0.5 mg/ml of *o*-phenylenediamine dihydrochloride (OPD, Sigma, St Louis, MO) with 10 µl of H₂O₂ in citrate buffer. The reactions were stopped by the addition of 50 µl of 3 M HCl to each well. The concentration of non-specific antibody was determined by comparison to a standard curve generated with unlabelled purified isotypes. Absorbance values were read at 492 nm in an automatic ELISA reader.

Western blot

The SDS–PAGE (12%) was run with 50 µg of LUC-*L. infantum* amastigotes total protein extract in each lane. After migration proteins were transferred onto a nitrocellulose membrane, using transfer buffer (10% Tris-glycine 10×, 20% of ethanol 100%). Transfer was performed at 80 mA for 75 min at room temperature (RT). Membranes were then saturated for 1 hr with a solution of PBS (0.01 M pH 7.4) complemented with 5% fat dehydrated milk, during 1 hr at RT. The first antibody (sera from LmSIR2-treated mice, sera from PBS-treated mice or preimmune sera) was incubated overnight at 4°, with agitation, and then washed three times in 0.05% PBS–Tween for 15 min at RT and two times in PBS for 10 min. Horseradish peroxidase (HRP)-labelled goat antibodies to mouse IgG were then added at a dilution of 1 : 5000 and membranes were incubated for 1 hr at RT with agitation, followed by washing procedures as above. At last, it was revealed with ECL Western blotting analysis system.

Immunofluorescence assays

L. infantum axenic amastigotes were fixed with 4% paraformaldehyde in PBS for 1 hr at room temperature. After several washes, the parasites were permeabilized with

0.1% (v/v) Triton-X-100 in PBS. Parasites were then incubated with a mouse immune serum to LmSIR2 or preimmune serum (control) diluted 1 : 100 in PBS and containing 1% bovine serum albumin (PBS–1% BSA). The secondary antibody used was Alexa Fluor 488 goat anti-mouse IgG diluted 1 : 100 in PBS–1% BSA (Molecular Probes, Eugene, OR). Washed parasites were mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA) and analysed with a fluorescent microscope (Axioskop-Carl Zeiss, Jena, Germany) at 1000 $\times$ magnification and images captured with a digital camera (Spot 2-Diagnostic Instruments, Sterling Heights, MI, USA) and the software Spot 3.1 (Diagnostic Instruments, USA).

Complement mediated lysis assay (CML)

CML assay was done according to Norris *et al.*²⁰ with some modifications. Briefly, cultured *LUC*-recombinant promastigotes or amastigotes were washed and resuspended in RPMI-1640 culture medium with 10% of BSA at 10⁷/ml. A 50- μ l portion of this suspension was incubated with a mouse anti-LmSIR2 immune serum for 1 hr, washed and incubated with rabbit complement (1 : 10) for an additional hour. All incubations were carried out at 37°. The complement lysis was determined by measuring the luciferase activity of the remaining intact parasites after washing. The percentage of lysis was determined as follows: killing percentage = 100 – [(luciferase activity after antibodies plus complement/luciferase activity after antibody plus inactivated complement) $\times$ 100].

In vitro macrophage infections

Peritoneal macrophages obtained from 6–8-week-old healthy male BALB/c mice were washed with prewarmed RPMI-1640 medium supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin and cultured at 2 $\times$ 10⁴ macrophages/well in 96-well test plates (TPP, Trasadingen, Switzerland). Non-adherent cells were removed by washing twice with prewarmed RPMI medium and macrophages were infected with cultured *LUC*-recombinant amastigotes (pretreated or not with sera for 1 hr at 37° with 5% CO₂) at a ratio of 5 : 1 amastigotes per macrophage for 2 hr at 37° with 5% CO₂. Non-internalized parasites were removed by gently washing, and culture was maintained for 24 hr. The sera were decomplexed by incubation at 56° for 1 hr in a water bath.

Luciferase activity

The luciferase activity of the *LUC*-recombinant parasites in the CML assay or in intracellular amastigotes isolated from infected macrophages was determined essentially as

described elsewhere.²¹ Values were expressed as relative light units (RLU).

Statistical analysis

The data were analysed using Student's *t*-test.

Results

B cells, but not T cells, express CD69 in response to LmSIR2 treatment

In previous studies we have examined the antibody response during human and canine *L. infantum* infections using defined recombinant *Leishmania* proteins (LmS3a, LmSIR2 and LimTXNPx¹⁶). The LmSIR2 was found to be among highly immunoreactive antigens. Thus, we thought it was reasonable to examine the effect of LmSIR2 on the cells of the immune system using a murine model.

To identify the cell populations responding to LmSIR2, we analysed the *in vitro* membrane expression of CD69, an early activation marker, by CD4⁺, CD8⁺ and B cells. As shown in Fig. 1(a), normal spleen cells from BALB/c mice, cultured in the presence of LmSIR2 for 20 hr, showed increased expression of CD69 marker on B cells compared to the unstimulated cells. Interestingly, no significant increase in CD69 surface expression was observed in the case of CD4⁺ and CD8⁺ T-cell populations when subjected to the same treatment. As a positive control, lipopolysaccharide (LPS) induced high expression of CD69 by B cells (Fig. 1a). Therefore, in order to rule out the possibility that LmSIR2 biological activity could be linked to endotoxin contaminants, additional experiments where polymyxin B was added to the culture were conducted. As shown in Fig. 1(b), the presence of polymyxin B was able to significantly abrogate the LPS activity whereas no effect on LmSIR2-induced up-regulation of CD69 expression by spleen cells could be demonstrated.

To further explore whether the activation of B cells by LmSIR2 requires T-cell dependent mechanisms, complementary experiments were done using BALB/c purified B splenocytes. LmSIR2-treated purified B cells showed significant increase of CD69 surface expression (95% CD69 positive B cells) when compared to the control non-treated cells (10% CD69 positive B cells). Positive control test using LPS as a triggering agent showed increased expression of CD69 on B cells. Interestingly, polymyxin B partially prevents LPS but not LmSIR2-induced CD69 expression (Fig. 1c). These data allow excluding the potential involvement of contaminating endotoxin in LmSIR2-induced up-regulation of CD69 expression by B cells. Similar results were obtained when using spleen cells from athymic-BALB/c mice (nude mice; data not shown). It is thus reasonable to assume that the LmSIR2 preferentially triggers the direct activation of B cells.

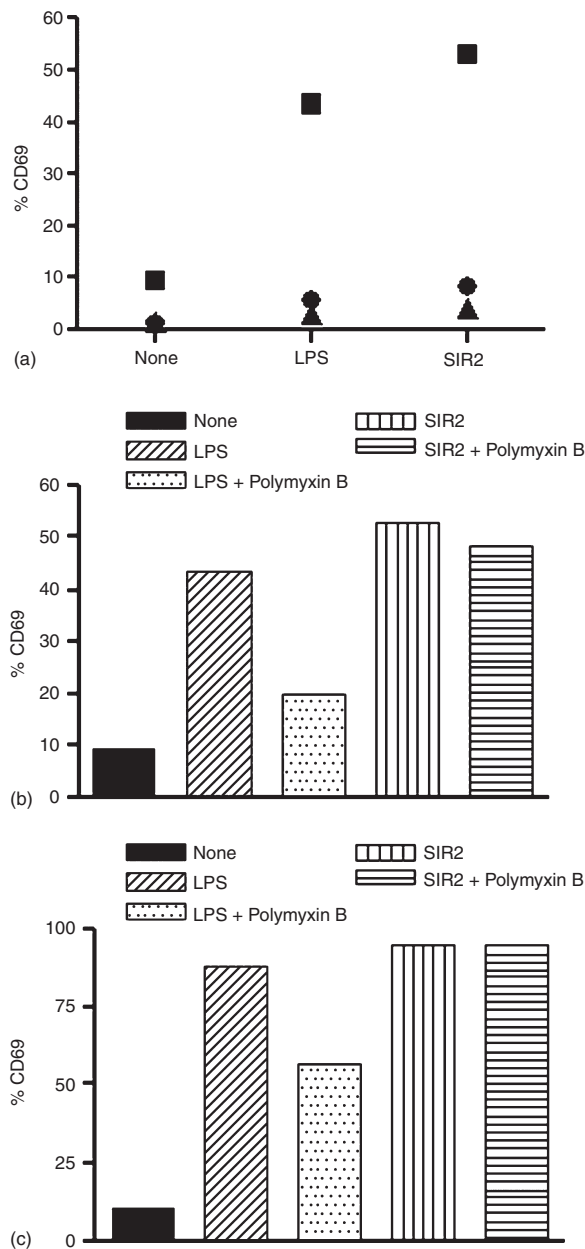


Figure 1. B cells express CD69 in response to LmSIR2. Expression of CD69 activation marker in spleen cells from BALB/c (a and b) and purified spleen B cells isolated from BALB/c mice (c) after culture with LmSIR2. A total of 2.5×10^5 cells per well were cultured in the presence of LmSIR2 (10 $\mu\text{g}/\text{ml}$) or LPS (10 $\mu\text{g}/\text{ml}$). In some experiments 10 μg of polymyxin B per ml was added to LmSIR2 and LPS. To determine the percentage of CD69 in B (■) cells or CD4 (●) or CD8 (▲) T cells, the different cell populations were positively gated. The results are from a representative experiment of three carried out independently.

LmSIR2 induces *in vivo* increased number of spleen B cells

Although LmSIR2 was able to stimulate the expression of CD69 on B cells, we failed to show *in vitro* LmSIR2-

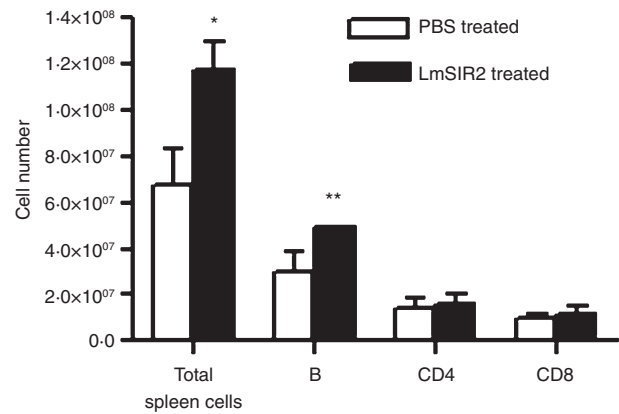


Figure 2. *In vivo* injection of LmSIR2 into BALB/c mice induced spleen B-cell proliferation. The percentage of B cells, CD4 and CD8 T cells in the spleen of untreated and LmSIR2 (20 $\mu\text{g}/\text{mouse}$)-treated BALB/c mice 72 h after the LmSIR2 *i.v.* injection were determined by flow cytometric analysis. The number of each spleen cell subpopulation was calculated based on the total viable cells determined by trypan blue exclusion and the percentage of cell bound mAb to the specific cell surface marker (anti-CD4, anti-CD8 and anti- μ). The data represent the mean and the standard deviations of three animals analysed individually and are representative of two independent experiments. Statistically significant differences between untreated and LmSIR2 treated mice are indicated: * $P < 0.01$; ** $P < 0.05$.

induced proliferation of B cells. Indeed, no proliferation of total spleen cells or purified B cells could be evidenced upon incubation with LmSIR2. This may suggest that, besides the LmSIR2 activity, others signals might be necessary to provide a fully B-cell activation and proliferation.²²

In order to analyse the *in vivo* effect of LmSIR2 on the cell populations, the protein was injected intravenously (*i.v.*) into BALB/c mice and 72 hr later the levels of cell populations in the spleen were determined by FACS analysis using mAb against cell surface markers. As shown in Fig. 2, large increases in total splenocytes were seen in the case of LmSIR2-injected mice when compared to the PBS-injected mice. Analysis of subpopulations of splenocytes showed striking increase of B-cell number (1.6-fold increase compared to the control mice, $P < 0.05$) whereas no significant difference in total CD4 or CD8 cells could be evidenced when compared to the control mice. Because of the fact that the total increase in the number of spleen cells is much greater than that of B cells, we can not rule out that other cell populations (*i.e.* macrophages, dendritic cells) are responding to LmSIR2. It is also possible that the increased number of splenic B cells might be the result of migration of B cells from lymph nodes or peripheral blood, not cell division. Taken together these observations strengthen the notion that B cells are targets of LmSIR2.

Table 1. Total serum immunoglobulins of BALB/c and BALB.nude mice 14 days after the last LmSIR2 injection

		Total serum Ig ($\mu\text{g/ml}$)	
		IgM	IgG
BALB/c	PBS-treated	301.5 $\pm$ 70.3	1929.6 $\pm$ 61.4
	LmSIR2-treated	649.0 $\pm$ 197.1	4830.9 $\pm$ 520.9
		$P = 0.1436$	$P = 0.0050$
BALB/c nude	PBS-treated	380.1 $\pm$ 33.1	464.4 $\pm$ 42.9
	LmSIR2-treated	570.4 $\pm$ 79.1	453.5 $\pm$ 11.3
		$P = 0.0014$	$P = 0.9670$

LmSIR2 injection into BALB/c mice promotes a strong humoral response with specific immunoglobulin secretion

To further examine the effects of LmSIR2 on the B-cell response, we determined the levels of the different isotypes in the sera of mice that received three i.p. injections of LmSIR2 at 7 days interval, and control mice, which received PBS. A strong humoral response, as evidenced by increased levels of total immunoglobulins, was found in the sera of BALB/c mice injected with LmSIR2 compared to the sera from PBS-treated mice (Table 1). Statistical analysis showed significant differences between PBS and LmSIR2-treated mice only in the case of IgG antibodies ($P = 0.005$). Moreover, analysis of IgG antibodies showed significant increase in all IgG isotypes, being the higher increases in the IgG1 and IgG2a subclasses in the sera of mice which received LmSIR2 protein compared to the controls ($P < 0.05$, Fig. 3a).

Further, we explored the specificity of the immune response. As indicated in Fig. 3(b), mice treated with LmSIR2 developed IgG1 and IgG2a antibodies which recognized specifically LmSIR2. No reactivity could be seen when using heterologous antigens (i.e. KLH or myosin) or another recombinant *Leishmania* ribosomal protein namely LmS3a. However, very low reactivity was observed when using *L. infantum* total extracts in the ELISA test. This is not surprising given the specificity of the serum and that SIR2 protein is expressed at low levels whatever the *Leishmania* strain extract used (data not shown).

These observations indicate that LmSIR2 when injected without adjuvant in BALB/c mice induces a strong B-cell activation and differentiation leading to a strong humoral response with specific antibodies secretion.

LmSIR2 induce B-cell proliferation by a T-cell independent mechanism

The above data therefore suggested that LmSIR2 when administrated *in vivo* to BALB/c mice stimulates the

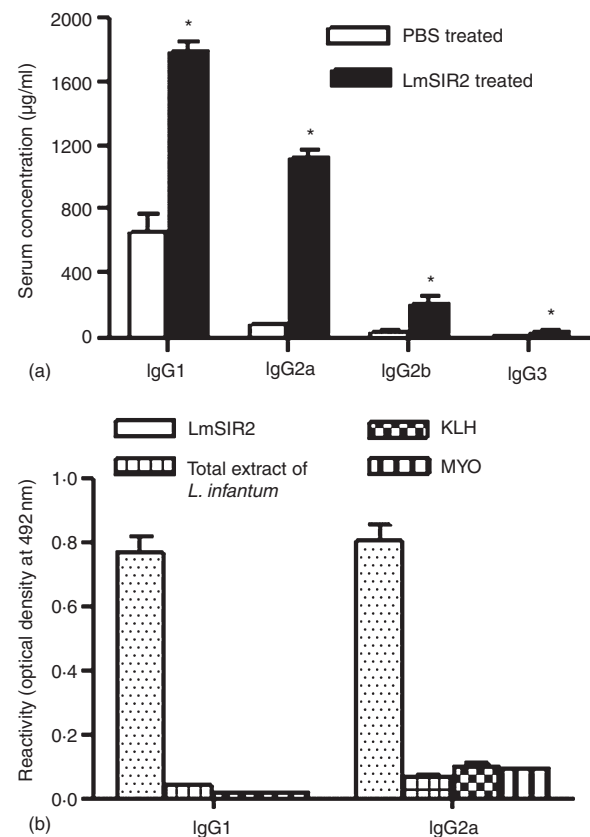


Figure 3. Humoral immune response of BALB/c mice 15 days after the last LmSIR2 injection. Groups of three mice were immunized i.p. with LmSIR2 as indicated in Materials and methods. (a) Levels of total IgG1, IgG2a, IgG2b and IgG3 in the sera of LmSIR2-treated and untreated BALB/c mice were quantified by ELISA in comparison to standard curves using purified mouse IgG1, IgG2a, IgG2b and IgG3, respectively. The asterisk indicates a statistically significant difference ($P < 0.05$) between untreated and LmSIR2-treated mice. (b) IgG1 and IgG2a antibodies levels in the sera of LmSIR2-treated BALB/c mice reacting against LmSIR2, *L. infantum* total extract, key-hole limpet hemocyanin (KLH) and myoglobin (MYO) were determined by ELISA. Sera were diluted 1/100 in PBS–1% gelatin. The data represent the mean and the standard deviations of three animals analysed individually and are representative of two independent experiments.

B, but not the T lymphocytes, inducing B-cell differentiation. To further confirm that LmSIR2 was activating B cells, but not T cells, similar experiments were performed in nude mice using LmSIR2 that was passed through an EndoTrap®red column to eliminate residual endotoxin contaminants. The data shown in Table 1 demonstrated that nude mice responded to LmSIR2 injection by producing significant levels of IgM antibodies when compared to PBS-injected nude mice ($P = 0.0014$). Moreover, the IgM antibodies when reacted in ELISA test showed specific recognition of LmSIR2 (Fig. 4). Altogether, these data suggest that LmSIR2 triggered significant B-cell differentiation and secretion of specific antibodies.

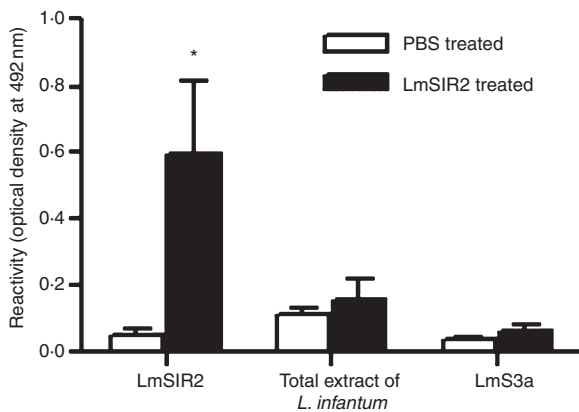


Figure 4. Levels of specific IgM anti-LmSIR2 in the sera of BALB-nude mice. Sera from LmSIR2 and PBS injected mice were reacted in ELISA assay against *L. infantum* total extract, LmS3a or LmSIR2. Statistically significant differences between sera from LmSIR2-treated and control mice were observed: * $P < 0.005$.

Anti-LmSIR2 antibodies bind to the amastigote surface

The above observations demonstrated that LmSIR2 immunization of mice induced a strong specific antibody response. Thus, we examined the reactivity of anti-LmSIR2 mice immune serum against *L. infantum* axenic amastigotes carrying a luciferase encoding gene (*LUC-L. infantum*). As shown in Fig. 5(a), sera from mice immunized with LmSIR2 recognized the corresponding antigen (MW $\approx$ 52 000) in *LUC-L. infantum* extracts whereas no reactivity could be seen when using pre-immune sera or the sera from PBS-injected mice. Moreover, indirect immunofluorescence assays showed a positive labelling in vesicles and in the flagellar pocket zone (Fig. 5b), that may indicate protein secretion from the parasite.²³

We then analysed by FACS whether a binding of the anti-LmSIR2 antibodies was detectable. The results shown in Fig. 5(c) indicated that indeed, anti-LmSIR2 bound to the amastigote. The level of binding was approximately three times higher when using sera from LmSIR2-immunized mice compared to preimmune sera (MFI = 45 and 15, respectively).

Anti-LmSIR2 antibodies induce complement mediated lysis and inhibit amastigote development inside macrophages *in vitro*

The above data indicate that LmSIR2 epitopes are expressed on the parasite surface and are accessible to the antibodies. Therefore, we thought it was reasonable to determine the ability of sera from LmSIR2-immunized mice to lyse *in vitro* *LUC-L. infantum* axenic amastigotes. As shown in Table 2, the percentage of killing of amasti-

gotes was between 21 and 45% depending on the concentration of serum used. As a control, preimmune serum was unable to support lysis of the parasites even at high concentration. This was also the case for a IIIG4 mAb against LmSIR2,¹⁴ which showed no capacity to induce parasite lysis. This might be the result of the fact that IIIG4 mAb, being of the IgG1 subclass, fixes complement poorly. Furthermore, the anti-LmSIR2 immune serum was also found to be able to induce the lysis of *LUC-L. infantum* promastigotes (data not shown).

We also tested whether the anti-LmSIR2 immune sera were able to modulate *in vitro* amastigote-macrophage interaction. Mouse peritoneal macrophages were used as *in vitro* model to measure infection with *LUC-L. infantum* axenic amastigotes in the presence of heat-inactivated anti-LmSIR2 immune serum. Thus, peritoneal macrophages were incubated with *LUC-L. infantum* axenic amastigotes at a ratio of five parasites per macrophage in the presence of 1 : 10 dilution of preimmune serum or sera from LmSIR2 and PBS-injected mice. Twenty-four hr later, the level of infection was determined. As shown in Fig. 6 there was a significant inhibition of amastigote development inside macrophages in the presence of heat-inactivated anti-LmSIR2 immune serum when compared to the control preimmune sera or sera from PBS-injected mice. These data suggest that anti-LmSIR2 sera in the absence of complement can reduce intracellular parasite development.

In preliminary immunization experiments, two groups of four mice each were injected i.p. with or without 50 μ g of LmSIR2 in 200 μ l of PBS three times at 7 day intervals. Fifteen days later, they were infected i.p. with 10^8 WT promastigotes. At 15 days postinfection, a significant decrease (≈ 1.5 -log reduction; $P = 0.03$) of parasite load was observed in the spleen of LmSIR2-immunized mice in comparison to the control (PBS-injected mice).

Discussion

In the current study, we demonstrated that the *Leishmania* cytosolic nicotinamide adenine dinucleotide-dependent deacetylase, LmSIR2, could directly activate B lymphocytes (but not T cells) from normal mice. However, although we were unable, though, to show the *in vitro* proliferation of spleen cells or purified B cells upon incubation with LmSIR2, *in vivo* administration of LmSIR2 i.v. into BALB/c mice triggered the differentiation of B cells, demonstrating therefore that LmSIR2 could serve as an important activation signal *in vivo*. Indeed, *in vivo* injection of LmSIR2 alone without adjuvant into BALB/c mice or nude mice induced the synthesis of IgG and IgM antibodies, respectively, thereby indicating that B cells were able to undergo differentiation into antibody-secreting cells. Several experiments demonstrated that the B lymphocytes were responding directly

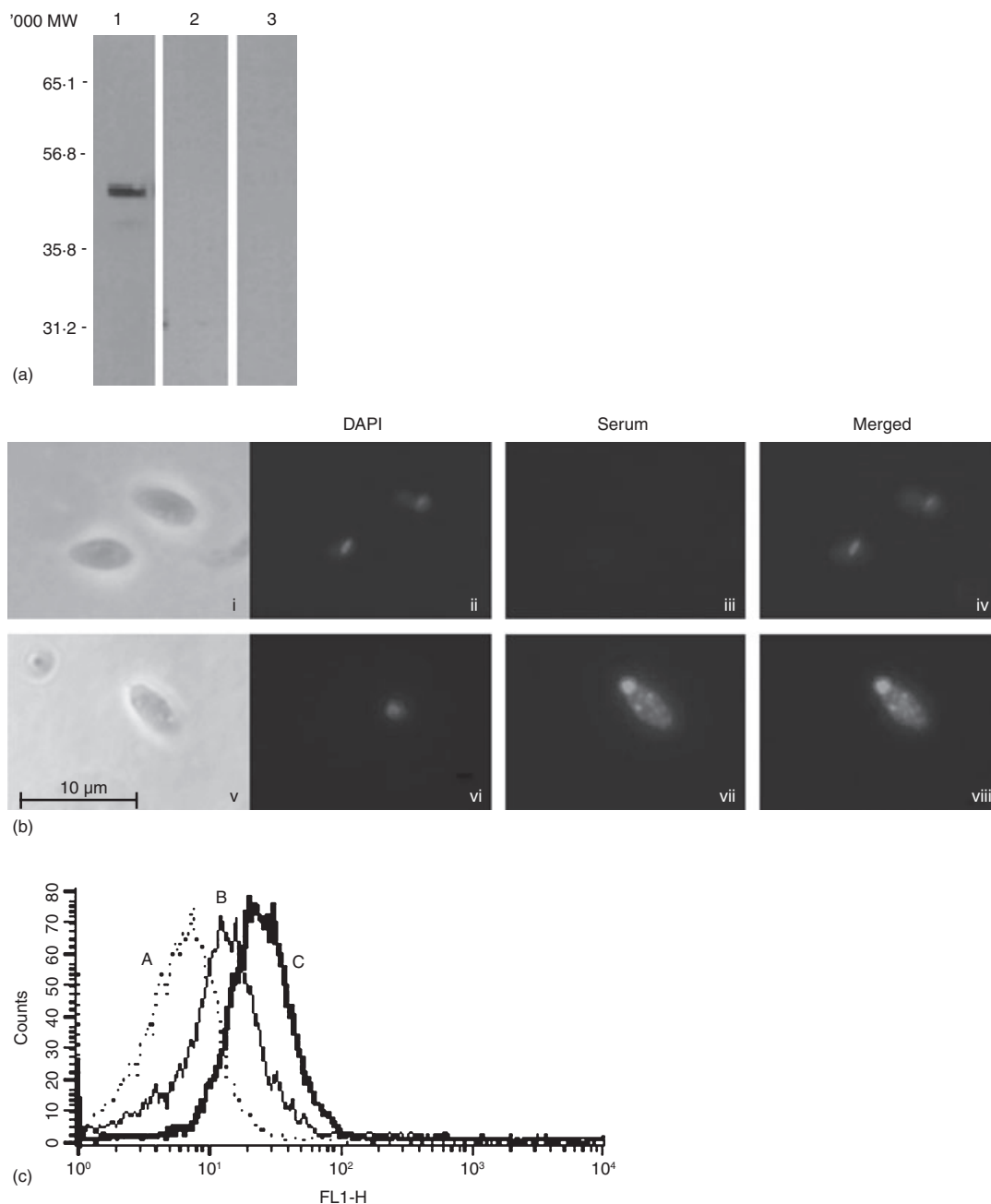


Figure 5. (a) Western blot of *LUC-L. infantum* amastigotes total protein extract reacted with sera from LmSIR2-treated BALB/c mice (1); sera from PBS-treated mice (2); preimmune sera (3). (b) Immunofluorescence using a preimmune serum (ii–iv) and serum from LmSIR2-treated mice (vi–viii) reacted with *L. infantum* axenic amastigotes. Parasites were photographed at 1000×. Contrast phase pictures of the preparations are also included (i, v). (c) FACS analysis of anti-LmSIR2 antibodies binding to *LUC-L. infantum* amastigote surface. Parasites were incubated with mouse immune serum to LmSIR2, followed by FITC-conjugated goat antimouse IgG (C). As controls, amastigotes were incubated with FITC-conjugated goat antimouse IgG (A) or preimmune serum followed by FITC-conjugated goat antimouse IgG (B).

to LmSIR2 and not to endotoxin contaminants. The production of antibodies in nude mice that lack T cells is direct evidence of the capacity of LmSIR2 to induce a humoral response by a T-cell independent mechanism. Because the nude mice are athymic, these animals cannot produce T-cell cytokines for isotype switching of

antibodies. Indeed, in the case of BALB/c mice, an immunoglobulin isotype switch occurs, as shown by the high production of all IgG isotypes. Taken into account that T-cell independent antigens stimulate mainly the IgM production, being the IgG secretion in lower amounts, restricted to the IgG3 isotype²⁴ it is tempting to speculate

Table 2. Complement-mediated lysis of *L. infantum* axenic amastigotes

Treatment	% Lysis at a dilution of ^a	
	1 : 5 ^b	1 : 10 ^b
Anti-LmSIR2 mouse immune serum	45	21
Monoclonal Anti-LmSIR2 (IIIG4)	2	1
Pre-immune serum	0	0

^aThe percentage of lysis is the mean of triplicate determinations of a representative experiment from three carried out independently.

^bA optimized 1 : 10 dilution of rabbit complement was used with different anti-LmSIR2 immune serum dilutions.

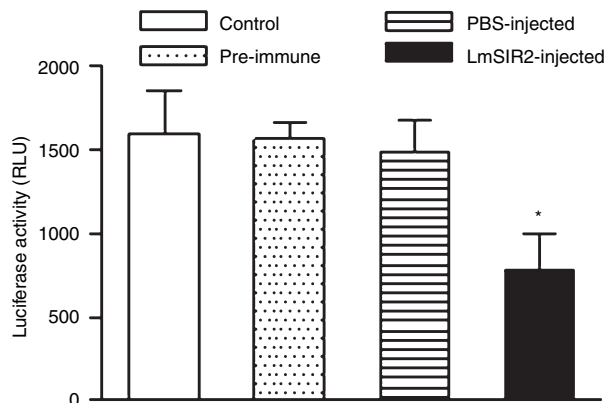


Figure 6. Effect of anti-LmSIR2 antibodies on amastigote development inside macrophages. A total number of 2×10^4 BALB/c peritoneal macrophages were infected at a ratio of 5 : 1 *LUC*-amastigotes/macrophage and the number of intracellular amastigotes was determined after 24 h by measuring luciferase activity. Prior to infection, the amastigotes were incubated with indicated sera samples (1 : 10 dilution) or control (no serum). The asterisk indicates a statistically significant difference (* $P < 0.01$) in comparison with the values obtained using normal serum. The results are from a representative experiment of three carried out independently.

that LmSIR2 has dual functions *in vivo*, being able to act as a T-cell independent or a T-cell dependent antigen, which could explain its enhanced immunogenicity. However, the classic division of antigens in these two categories is not absolute. In fact, previous studies in hepatitis B and vesicle stomatitis virus have already reported the existence of antigens that can induce B-cell activation and proliferation through both T-dependent and -independent mechanisms.^{25,26} However, other possible interpretations can be made. It has been proposed that in immunocompetent mice, after activating the B cells, some T-independent antigens require a 'second signal' in order to develop an IgG secretory response²⁷ and this could also be the case for LmSIR2. However, so far the support for the 'second signal hypothesis' has been elusive. This may

be due to the high number of candidates that are capable of giving this potential second signal.²⁷ Thus, these responses might be dependent on differentiation-inducing factors produced by antigen non-specific cells.²⁸ Natural killer cells and macrophages were already been referred to be involved in this process, as much for the release of cytokines after activation as for the mobilization of T cells and thereby their derived cytokines.²⁹ Recently, two tumour necrosis factor (TNF) family ligands, BAFF (B-cell activation factor of the TNF family) and APRIL (a proliferation-inducing ligand) were implicated in several immunological phenomena, such as peripheral B-cell survival, T-cell independent antibody isotype switching, and the induction of self-reactive B cells.³⁰ These ligands are expressed in macrophages, dendritic cells and T cells, and their up-regulation caused by cytokines produced through the activation of the immune system provides survival signals, and also may contribute to class switch recombination in B cells activated by T-independent antigens.³¹ Thus, so far, the mechanism by which LmSIR2 is capable of inducing immunoglobulin isotype switching in the presence of T cells but not in their absence (BALB/c mice and BALB.nude mice, respectively) is still unknown. Further studies will have to investigate in detail the mechanisms leading to the LmSIR2-induced isotype switching *in vivo*.

It is well known that *Leishmania* spp. release a large number of molecules that could act as mitogenic substances inducing polyclonal lymphocyte responses and consequently a general lack of specificity of antibodies and T-cell responses during the infection. Indeed, soluble parasite-derived antigens from *L. major* and *L. donovani* are mitogenic and trigger the production of immunoglobulins with autoantibody activity.⁶ Thus, crude extracts of *L. donovani* contains components that cause strong *in vitro* polyclonal activation of hamster spleen cells.⁷ Moreover, an excreted factor derived from the culture medium of *L. major* was found to suppress concanavalin A-induced polyclonal activation of mouse T cells.³² Furthermore, in previous reports, we have identified a *Leishmania* gene encoding a protein sharing significant homology to mammalian ribosomal proteins S3a named LmS3a exhibiting dual activity being stimulatory and inhibitory towards T and B cells, respectively.³³ The investigations on the immunogenicity of LmS3a have revealed that *in vivo*, a single injection of the recombinant protein without any adjuvant into mice induced a quick increase in the number of B cells and the production of high levels of immunoglobulins, mainly of IgM isotype. The IgM response was mostly unrelated to the antigens present in the total parasite extracts or to the protein itself. These observations contrasted with the *in vivo* LmSIR2 activity on B cells. Indeed, although the protein triggered B-cell differentiation, the antibodies produced reacted specifically against

SIR2 and not other heterologous antigens such as myosin or KLH, and were able to induce the complement-mediated killing of amastigotes and inhibition of their multiplication inside macrophages. Indirect immunofluorescence assays of *L. infantum* axenic amastigotes with sera from LmSIR2-immunized mice showed the presence of SIR2 protein in vesicles and in the flagellar pocket zone, already known to be filled with secretory material;²³ this being therefore in agreement with our previous observations.¹⁸ Moreover, using a highly sensitive radiolabelled immunoprecipitation technique, we observed that SIR2 is among the parasite excreted-secreted antigens (unpublished data). Results from the *in vitro* macrophage infections with *LUC-L. infantum* amastigotes, the presence of anti-LmSIR2 sera may indicate a potential role of SIR2 in the binding, internalization and/or multiplication of the parasite in the macrophage.

Studies on the molecular mechanisms of parasite entry into macrophages have led to the identification of several candidate receptors facilitating multiple routes of entry.³⁴ Indeed, internalization of promastigotes into macrophages has been shown to be mediated by macrophage membrane proteins such as the mannose receptor,³⁵ the fibronectin receptor,³⁶ the Fc receptor (FcR),³⁷ and the complement receptors such as CR1 (CD35) and CR3 (CD11b/CD18).^{35,38} Thus, the *in vitro* modulation of macrophage infection by anti-LmSIR2 antibodies may suggest possible roles of SIR2 in the internalization process of the amastigote and/or further multiplication of the parasite.

Evasion from the host complement system is one of the strategies used by *Leishmania* parasites to avoid host immune defence.³⁹ Indeed, metacyclic promastigotes and amastigotes are relatively resistant to direct serum killing.⁴⁰ However, previous studies have shown that antibodies that were able to bind to living parasites and lyse them in conjunction with complement were associated with host protection.²⁰ Thus, one can assume that, the production of antibodies capable of enhancing complement cytotoxicity towards the amastigote stage might be, working in co-operation with the cellular immunity, an important requirement for effective antiparasitic immunity.⁴¹ Given that the anti-LmSIR2 sera induced complement-mediated lysis of amastigotes and promastigotes, one may speculate that a LmSIR2 immunization can be seen as capable of protecting the host against infection and disease progression.

Although it has been reported that the antibodies may play a role in the host protection mechanisms against experimental leishmaniasis¹² a more recent study has shown that antibodies could exert deleterious effects on the host. In fact, by using B cell-mutant mice and genetically modified mice lacking circulating antibodies infected with *L. amazonensis* and *L. pifanoi*, the authors

reported that these mice developed barely detectable lesions compared to control BALB/c mice.⁴² Reconstitution of the B cell-mutant with the immune anti-*Leishmania* serum increased the pathological processes in the otherwise non-susceptible mice. Therefore, preliminary BALB/c immunization experiments were conducted using LmSIR2 protein. A significant decrease of parasite load in the spleen of LmSIR2-immunized mice was observed in comparison to non-immunized control mice at 2 weeks postchallenge infection. Thus, it is reasonable to suggest that anti-LmSIR2 antibodies may in part play a role in protective immune mechanisms rather than exacerbating the disease. Further studies using different doses and routes of immunization are needed to explore further the protective role of the LmSIR2 molecule.

The LmSIR2 protein belongs to a highly conserved family of closely related proteins in both prokaryotic and eukaryotic species.⁴³ Historically, the biological significance of SIR2-like proteins was attributed to the histones' deacetylation, leading to chromatin condensation and transcriptional silencing.⁴⁴ However, diverse cellular localizations were found among SIR2 homologues, suggesting that these enzymes have other physiological substrates than histones and thus several biological functions inside the different organisms. Indeed, several roles have been attributed to SIR2-related family of proteins including cell cycle progression and chromosome stability,⁴⁵ DNA-damage repair,⁴⁶ life span extension in yeast⁴⁷ and in *Caenorhabditis elegans*.⁴⁸ To our knowledge, this report is the first description of a protein belonging to this large family, which is among the *Leishmania* cytosolic and secretory products that proved to have a role in the regulation of the immune response through its capacity to trigger preferentially B-cell effector functions. The high degree of homology within this family of proteins, and the fact that homologues have been found in mouse⁴⁹ and human⁵⁰ has led us to perform additional experiments in order to verify the possibility of the existence of SIR2 cross-reactive epitopes on the mouse and human cells that could be recognized by the sera from LmSIR2-immunized mice. Using different approaches (Western blot, ELISA, immunoprecipitation of [³⁵S]methionine-labelled mouse spleen cells) no such cross-reactivity was found, arguing for the *Leishmania* specificity of the antibodies produced by LmSIR2 immunization (data not shown).

In summary, our present results show that LmSIR2 is a potent B-cell modulatory factor both *in vitro* and *in vivo*. Following the injections of LmSIR2 without adjuvant a selective B-cell response was induced resulting in a surprisingly parasite-specific production of antibodies, which were lytic in co-operation with the complement, and restrain the capacity of the parasites to infect macrophages. There is strong evidence that an ideal vaccine, especially against visceral leishmaniasis, may require the

induction of both humoral and cellular branches of the immune system, for maximum efficiency.¹¹ In that view, one can consider LmSIR2 to be among vaccination candidate, taking into account the safety and the strong response when delivered in a low dose. Overall, these data add LmSIR2 to the list of *Leishmania* antigens that could specifically stimulate the immune system of the host and suggest that LmSIR2 could be among the parasite molecules to be used to design an optimal multicomponent vaccine to control *Leishmania* infection.

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