Universitat Autònoma de Barcelona

DRUG REPURPOSING OF BIOENERGETIC MODULLATORS: USE IN TREATMENT AND VACCINATION OF PROTOZOAN PARASITIC DISEASES

Alba Martínez Flórez

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By

Alba Martínez Flórez

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We certify that the dissertation entitled "Drug repurposing of bioenergetic modulators: Use in treatment and vaccination of protozoan parasitic diseases" submitted by **ALBA MARTÍNEZ FLÓREZ** in partial fulfilment of the requirements for the Degree of Doctor of Philosophy was carried out under my supervision and I authorize the submission to undertake its oral defense.

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I, perquè així consti, signem aquest document,

Dra. Alhelí Rodríguez Cortés
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ACRONYMS

1,3BPG	1,3-biphosphoglycerate
2DG	2-deoxy-D-glucose
3BP	3-bromopyruvic acid
3PG	3-phosphoglycerate
ACTs	Artemisinin-based combination therapies
ADP	Adenosine diphosphate
AmB	Amphotericin B
AMPK	AMP-activated protein kinase
APCs	Antigen Presenting Cells
ATP	Adenosine triphosphate
BSL2	Biosecurity level 2
BZD	Benznidazole
CanL	Canine leishmaniasis
CL	Cutaneous leishmaniasis
CMI	Cell mediated immunity
CQ	Chloroquine
CTLA	Crude Total Leishmania Antigen
DALYs	Disability-adjusted life years
DCA	Dichloroacetic acid
DCs	Dendritic cells
DHA	Dihydroxyacetone
ECACC	European Collection of Cell Cultures
ELISA	Enzyme-linked immunosorbent Assay
Em	Emission
EU	ELISA units
Ex	Excitation
FBS	Fetal bovine serum
FoxP3	Fork head box P3
G3P	Glyceraldehyde 3-phosphate
G6P	Glucose-6-phosphate
G6PD	Glucose-6-phosphate dehydrogenase deficiency
GAPDH	Glyceraldehyde phosphate dehydrogenase
Glc	Glucose
GLA-SE	Glucopyranosyl lipid A
HAT	Human African trypanosomiasis
HIF-1	Hypoxia induced factor 1
HK	Hexokinase
IC_{50}	Inhibitory concentration 50
IFN-γ	Interferon γ
IgG	Immunoglobulin G
IL-x	Interleukin-x
iNOS2	Inducible Nitric Oxide Synthase
IP	Intraperitoneal
IPT	Intermittent presumptive treatment
ISPF	1-phenyl-1,2-propanodione-2-oxime
KMPII	Kinetoplastid Membrane Protein-11
LACK	*Leishmania* homologue of receptors for Activated C kinase
LN	Lymph node
LND	Lonidamine
MCL	Mucocutaneous leishmaniasis
MET	Metformin
MHC	Major histocompatibility complex
M1	Classically activated Macrophages
M2	Alternatively activated Macrophages
mTOR	Mechanistic target of rapamycin
MTT	Thiazolyl Blue Tetrazolium Bromide
NFX	Nifurtimox
NTDs	Neglected Tropical Diseases
ODs	Optical densities
OXPHOS	Oxidative phosphorylation
P4HA	Procollagen-proline dioxygenase

PAPLE22	Potentially Aggravating Protein of *L. infantum*
PBS	Phosphate buffered saline
PDH	Pyruvate dehydrogenase complex
PDK	Pyruvate dehydrogenase kinase
PKDL	Post-kala-azar dermal leishmaniasis
PMA	Phorbol 12-myristate 13-acetate
PPP	Pentose Phosphate Pathway
PSS	Physiologic saline solution
qPCR	Quantitative Polymerase Chain reaction
RT-qPCR	Real time- qPCR
RBCs	Red blood cells
RLU	Relative Light Units
ROS	Reactive oxygen species
SIR	Sirolimus
SUR	Suramin
TCA	Tricarboxylic acid
Tfh	Follicular helper cells
TGF-β	Transforming growth factor β
Th	T helper cell
TLRs	Toll like receptors
TMB	Tetramethylbenzidine
TNF-α	Tumor necrosis factor α
Treg	T regulatory cell
TRYP	Tryparedoxin Peroxidase
VL	Visceral Leishmaniasis
WHO	World Health Organization

ABSTRACT

Leishmaniases, African and American trypanosomiases and malaria are parasitic diseases that constitute a major global health problem. The increasing number of drug-resistances to their current treatments, toxicity cases and the health assistance often required for their administration, makes it urgently necessary to develop efficient vaccines for humans and new affordable therapies, easy to apply and resistant to harsh storage conditions. Due to the fact that these diseases share similar metabolic requirements with better studied diseases, we chose drug repurposing as a potentially effective approach against them. With this purpose, six different compounds used in anti-cancer research —dichloroacetate (DCA), 3-bromopyruvate (3BP), 2-deoxy-D-glucose (2DG), lonidamine (LND), metformin (MET), and sirolimus (SIR)— were selected according to their ability to modulate energy production and proliferation related metabolic pathways. The aim of this study was to validate the suitability of these bioenergetics modulators for the management of visceral leishmaniasis, malaria and African and American trypanosomiasis as a treatment, or as a preventive tool by enhancing the protective power of a vaccine against *L. infantum*.

The effectiveness of these compounds was first evaluated on *in vitro* models of each parasite — Chagas disease (*Trypanosoma cruzi*), human African trypanosomiasis (*Trypanosoma brucei*), visceral leishmaniasis (*Leishmania infantum*) and malaria (*Plasmodium falciparum*)—. *L. infantum* promastigotes were not susceptible to these compounds, whereas *L. infantum* intracellular amastigote growth was dose-dependently reduced by 3BP (IC_{50} = 17.19 µM) and DCA (IC_{50} = 631.5 µM). In the *T. brucei in vitro* model all the tested compounds, with the exception of 2DG, affected parasite survival with IC_{50} values of 1.24 mM for DCA, 76.57 µM for 3BP, 26.76 µM for LND, 2.14 µM for SIR, and 17.30 mM for MET. In the case of *T. cruzi*, DCA, 3BP, 2DG, LND, and MET showed parasite-killing activity with IC_{50} values of 27.07 mM, 27.63 µM, 7.27 mM, 78.37 µM, and 18.48 mM, respectively. For *P. falciparum* DCA (IC_{50} = 5.39 mM), 2DG (IC_{50} = 4.19 mM), LND (IC_{50} = 209.13 µM), MET (IC_{50} = 1.32 mM), and SIR (IC_{50} = 2.50 µM), showed antiplasmodial activity. These results reinforce the hypothesis that drugs with proven efficacy in the treatment of cancer by interfering with energy production might be useful in treating threatening parasitic diseases and provide new opportunities for their repurposing.
However, when compounds that were effective in the *in vitro* approach were administered to the *in vivo* rodent models of these diseases, none of them contributed to disease management or parasite load control. Immunological analysis in the VL hamster model revealed a significant

downregulation of immune-activation in infected animals treated with DCA and 3BP, which may also contribute to treatment failure.

In the last chapter of this work, the suitability of sirolimus as an immunomodulatory compound to boost the activity of a preventive vaccine against VL was analyzed. Sirolimus is an already marketed compound that has been described to boost immune protection against different disease models. In our study, Syrian hamsters were treated with sirolimus concomitantly with the administration of a plasmid DNA vaccine carrying the *Leishmania* genes *LACK*, *TRYP*, *PAPLE22* and *KMPII*, and the subsequent response towards a *L. infantum* challenge was studied. Our results show that the DNA vaccine itself efficiently reduced the burden of parasites in skin (P = 0.0004) and lymph nodes (P = 0.0452), which was potentiated by SIR administration by also inducing parasitological protection in the spleen (P = 0.0004). The study of immune markers in spleen suggests that lower production of IFN-γ and the concurrent increase of FoxP3+ expression may be responsible for the protection mediated by the DNA vaccine that was potentiated by sirolimus.

RESUMEN

Las leishmaniasis, la tripanosomiasis Americana y Africana, y la malaria son enfermedades parasitarias que constituyen un importante problema de salud global que afecta mayoritariamente a países en desarrollo. El aumento del número de resistencias a sus tratamientos actuales, su toxicidad y la necesidad de asistencia sanitaria para la aplicación de los mismos reflejan la urgente necesidad de desarrollar vacunas eficaces y nuevos tratamientos económicos, fáciles de administrar y resistentes a condiciones de almacenamiento adversas. Basándonos en que estas enfermedades parasitarias comparten requerimientos metabólicos con patologías mejor estudiadas, proponemos el reposicionamiento de fármacos para tratarlas. Bajo esta premisa, seis fármacos de eficacia probada en la investigación contra el cáncer —dicloroacetato (DCA), 3-bromopiruvato (3BP), 2-deoxi-D-glucosa (2DG), lonidamina (LND), metformina (MET) y sirolimus (SIR)— fueron seleccionados por su habilidad para modular rutas metabólicas relacionadas con la producción de energía y proliferación. El objetivo de este estudio fue validar el uso de estos moduladores bioenergéticos para el control de la leishmaniasis visceral, malaria y tripanosomiasis americana y africana como tratamiento o como potenciadores de la protección de una vacuna frente a *L. infantum*.

Para ello, se evaluó la eficacia de estos compuestos en modelos *in vitro* de cada parasito —enfermedad de Chagas (*Trypanosoma cruzi*), tripanosomiasis Africana (*Trypanosoma brucei*), leishmaniasis visceral (*Leishmania infantum*) y malaria (*Plasmodium falciparum*)—. El 3BP y el DCA indujeron una reducción dosis-dependiente del crecimiento de los amastigotes intracelulares de *L. infantum* con IC_{50} de 17.19 µM y 631.5 µM, respectivamente. En el modelo *in vitro* de *T. brucei*, todos los compuestos testados, a excepción de 2DG, afectaron a la viabilidad del parásito: DCA (IC_{50} = 1.24 mM), 3BP (IC_{50} = 76.57 µM), LND (IC_{50} = 26.76 µM), SIR (IC_{50} = 2.14 µM), y MET (IC_{50} = 17.30 Mm). En el caso de los amastigotes intracelulares de *T. cruzi*, DCA, 3BP, 2DG, LND, y MET tuvieron efecto parasiticida con valores de IC_{50} de 27.07 mM, 27.63 µM, 7.27 mM, 78.37 µM, y 18.48 mM, respectivamente. DCA (IC_{50} = 5.39 mM), 2DG (IC_{50} = 4.19 mM), LND (IC_{50} = 209.13 µM), MET (IC_{50} = 1.32 mM), y SIR (IC_{50} = 2.50 µM), mostraron efecto antiparasitario sobre trofozoitos de *P. falciparum*. Estos resultados sugieren que estos fármacos podrían ser útiles para tratar estas enfermedades parasitarias. Sin embargo, cuando los compuestos eficaces en los modelos *in vitro* fueron administrados en modelos *in vivo* de roedor para cada una de las enfermedades, ninguno de ellos contribuyó al control de la

enfermedad o de la carga parasitaria. Los resultados obtenidos en el modelo de leishmaniasis visceral en hámster revelaron una disminución de la activación del sistema inmune en los animales tratados con DCA y 3BP, lo cual podría haber contribuido al fracaso del tratamiento.

Por último, se estudió la capacidad del SIR para potenciar el efecto protector de una vacuna frente a la leishmaniasis visceral en el modelo hámster. Para ello se administró SIR durante la fase de expansión y contracción del sistema inmune producido por una vacuna de DNA portadora de los genes *LACK*, *TRYP*, *PAPLE22*, y *KMPII* de *Leishmania*, y se estudió la respuesta frente al posterior desafío con *L. infantum*. Los resultados muestran que la vacuna de DNA indujo la reducción eficaz de la carga parasitaria en piel (P = 0.0004) y linfonodos (P = 0.0452), lo cual potenció la administración del SIR alcanzándose también protección parasitológica en bazo (P = 0.0004). El estudio de los marcadores inmunológicos en dicho órgano sugiere que la producción controlada de IFN-γ y el incremento en la expresión de FoxP3 podrían ser los responsables de la protección alcanzada.

INTRODUCTION

1. IMPACT OF PARASITIC DISEASES ON GLOBAL HEALTH

Socio-economic factors are strongly associated with disease and death. In our current world, more than 3 billion people in developing countries live with less than US$2, of whom around 6 million die every year due to the devastating HIV/AIDS, tuberculosis, and malaria. The severity of this situation is also aggravated by the concurrent infection by other infectious diseases, as poverty-stricken environments are also the perfect niche for their expansion (Hotez et al. 2006; Hochman et al. 2009). Among them, Neglected Tropical Diseases (NTDs) are a group of illnesses mainly affecting the poorest population in rural and impoverished areas in developing countries, thus reducing productivity and increasing social costs destined to treat and prevent these diseases, as well as hindering economic development. There are currently 17 diseases included in the NTD list of WHO, including several parasitic infections, such as hookworm disease and related intestinal helminth infections, schistosomiasis, lymphatic filariasis, onchocerciasis, and trachoma, as well as kinetoplastid infections like American and African trypanosomiasis (HAT) —termed Chagas disease and sleeping sickness, respectively—, and leishmaniasis (http://www.who.int/neglected_diseases/en/). Not all NTDs are highly lethal. Instead, most of them are chronic debilitating diseases. For that reason, the measurement of disability-adjusted life years (DALYs), which includes loss due to premature deaths and years living with a disability, is a better indicator to measure the real impact of these neglected conditions (Hotez et al. 2014). Globally, NTDs affect more than a billion people in 149 countries worldwide and are responsible for 26 million DALYs (World Health Organization 2015c). Because of their public health significance, amongst NTDs, it is worth emphasizing leishmaniases, Chagas disease, and HAT.

Several factors directly affect the epidemiological patterns of these parasitic infections and warrant special attention. Human and animal migrations increase the incidence of these diseases in countries neighboring conflict areas (Selby et al. 2013; Molaee Zadeh et al. 2014; Hayani et al. 2015), and also turn these conditions into emergent diseases in their new country of residence (Tanowitz et al. 2011). In addition, climate change may increase populations at risk (González et al. 2010; Moore et al. 2012; Caminade et al. 2014) through the proliferation of existing vectors (Poeppl et al. 2013) and exposing the parasites to potentially new vectors (Slama et al. 2014).

Special attention needs to be paid to HIV co-infection. Co-infection of HIV and *Leishmania*, or *Plasmodium* parasites seem to produce a mutual benefit for these pathogens (Xiao et al. 1998; Alvar et al. 2008; Noblick et al. 2011; Mock et al. 2012), and therefore, this condition has also brought back cases of parasitic diseases to countries where they were almost eradicated or at least not as common (Desjeux and Alvar 2003). HIV increases the risk of suffering from these parasitic diseases due to its immunosuppressive ability that leads to rapid recrudescence of infection and death (in the case of malaria and HIV co-infection, an estimated average of 2 million people die per year) (Kagira et al. 2011; Carolina Hernandez et al. 2014; World Health Organization 2015a).

Public health advances have substantially decreased the effect of these diseases in the last decades but more effort is still needed to eliminate them. The Sustainable Development Goals are part of the United Nations agenda to tackle sustainable economic, social, and environmental development, targeting poverty and inequality, as well as looking after global health and peace. Among these goals, is described the necessity to end the epidemics of HIV/AIDS, tuberculosis, malaria, and neglected tropical infections by 2030, providing a framework for investment in global health (United Nations 2017). As regards NTDs, the World Health Assembly also published in 2013 the WHA66.12, which targets NTDs to be prevented, controlled, eliminated, and eradicated (World Health Assembly 2013), sharing the script with the WHO Roadmap to eliminate some of these diseases by 2020, and achieving eradication by 2030 (World Health Organization 2015c). Additional research to develop new drugs, vaccines, diagnostics, and vector control agents and strategies are needed to achieve elimination. Recently, the fight against parasitic diseases has been awarded with the Nobel Prize in Physiology or Medicine. In 2015, this prize was shared by Ōmura and Campbell, and Tu, for their contribution to global health, with the discovery of avermectin and artemisinin for combating the neglected onchocerciasis and lymphatic filariasis, and malaria, respectively. This award emphasizes the importance of fighting parasitic infections and the global burden of deaths averted with proper treatments (Nobelprize.org 2015).

2. EPIDEMIOLOGY AND PATHOGENESIS

The present work is focused on leishmaniases, American and African trypanosomiasis, and malaria due to their public health significance.

2.1 Leishmaniasis

Leishmaniases are some of the most neglected diseases around the world. They are a major, although grossly underestimated, health problem, with over 350 million people being at risk in 88 endemic countries in tropical and subtropical biogeographic zones. Estimated annual incidence is over 2 million human cases resulting in more than 40 000 deaths per year, especially striking the poorest people in developing countries (Alvar et al. 2012; World Health Organization 2013).

2.1.1. Etiology and transmission

Table 1: Scientific classification of *Leishmania* parasites

LEISHMANIA	
Kingdom	Protista
Phylum	Euglenozoa
Class	kinetoplastida
Order	Trypanosomatida
Family	Trypanosomatidae
Genus	*Leishmania*
Species	*infantum* (*chagasi*), *donovani, panamensis, major, amazonensis*…

Protozoan parasites of the genus *Leishmania* are responsible for provoking the leishmaniases. So far, 53 *Leishmania* species have been described, and 20 of them are known to be pathogenic to humans (Akhoundi et al. 2016). Other natural hosts are canids, rodents, marsupials, and hyraxes, and therefore they play an important role as reservoirs for human infection (World health Organization 2016).

Leishmaniases are mainly transmitted by infected female sand fly vectors (Diptera, Psychodidae). There are more than 800 species of sand flies from which 90 belong to either the *Phlebotomus* genus (in the Old World) or the *Lutzomyia* genus (in the New World), which are able to mediate infection to humans (Akhoundi et al. 2016). Although not that common,

Leishmania infections due to laboratory accidents (Herwaldt 2001), organ transplantation (Sirvent-von Bueltzingsloewen et al. 2004; Singh and Sehgal 2010), vertical transmission (Meinecke et al. 1999; Boggiatto et al. 2011), and needle sharing among intravenous-drug users (Kaushik et al. 2011) have also been reported.

There are different clinical presentations of leishmaniases, with cutaneous leishmaniasis (CL) being the most common. CL is caused by *L. major*, *L. aethiopica* and *L. tropica*, among others, and has an estimated incidence of 0.7–1.2 million cases, most of them in Afghanistan, Iran, Syria, Algeria, Brazil, and Colombia. Mucocutaneous leishmaniasis (MCL) is mainly associated with *L. braziliensis* infections, but other cutaneous *Leishmania* species can also cause this clinical presentation. MCL has a low annual incidence and has a 90 % spread in South America, particularly in Bolivia, Brazil and Peru (World health Organization 2016). Visceral Leishmaniasis (VL), also known as kala-azar, is the most severe form of the disease with a fatal outcome if left untreated. From the total burden of cases of leishmaniases, 0.2–0.4 million cases are VL, which are mainly diagnosed in Brazil, Ethiopia, Sudan, South Sudan, India, and Bangladesh (Alvar et al. 2012). VL is caused by *L. donovani* and *L. infantum*, belonging to the *L. donovani complex*. It is believed that *L. donovani* is mostly transmitted from human to human (anthroponotic transmission) in the subtropical zones of Africa and Asia, while *L. infantum* is distributed in South America ecozones and in the Mediterranean Basin and follows a zoonotic cycle where the dog plays an important role as a domestic and peri-domestic reservoir, increasing the risk of infection of susceptible human populations cohabitating the same area (Boelaert et al. 2009; World Health Organization 2013; World Health Organization 2015c).

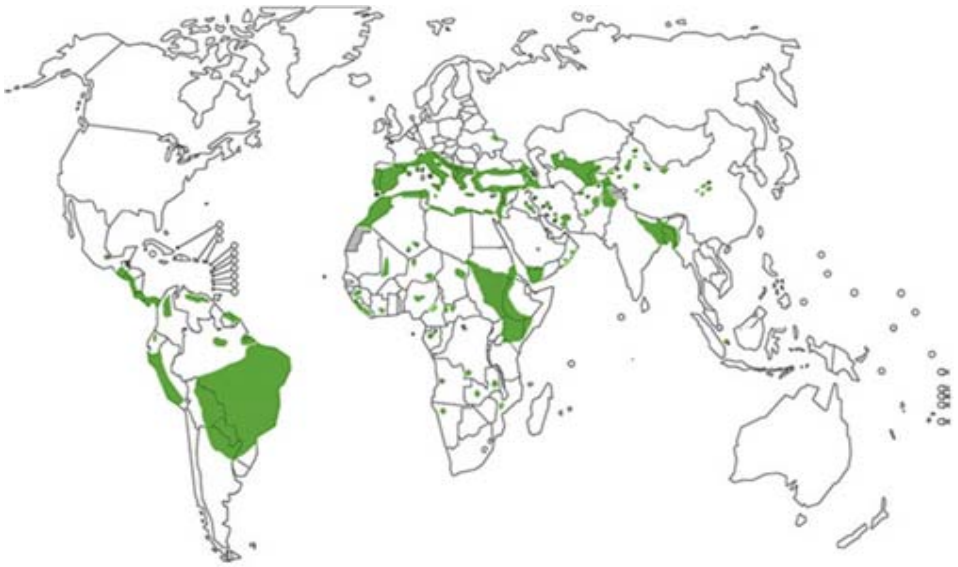


Figure 1: Geographic distribution of VL. Modified from WHO

2.1.2. Biological cycle of *Leishmania spp*.

When the blood-sucking female sand fly takes a meal, metacyclic promastigotes are inoculated in the vertebrate host. Inside the vertebrate host, *Leishmania* is an obligate intracellular parasite with macrophages being the widely used host cell. When the parasite is internalized by the macrophage, it remains in the endophagic vesicle, which fuses with lysosomes to become the parasitophorous vacuole, where *Leishmania* is adapted to grow in its low pH (McConville et al. 2007; Naderer and McConville 2008). In the parasitophorous vacuole, the metacyclic promastigote evolves to an amastigote, a highly replicative parasite form that starts to multiply and leads to the rupture of the macrophage, therefore releasing amastigotes that will be phagocytized by other host cells. New sand flies get infected when taking parasitized macrophages during a blood meal. Macrophages are digested in the invertebrate host's gut, amastigotes are released and transform into procyclic promastigotes. These parasites are able to divide in the invertebrate host's gut. From there, migration starts to the anterior midgut, where parasite maturation is induced (Gossage et al. 2003) and metacyclic promastigotes will be ready to be regurgitated during the next blood meal, completing the cycle (Centers for Disease Control and Prevention (CDC) 2013).

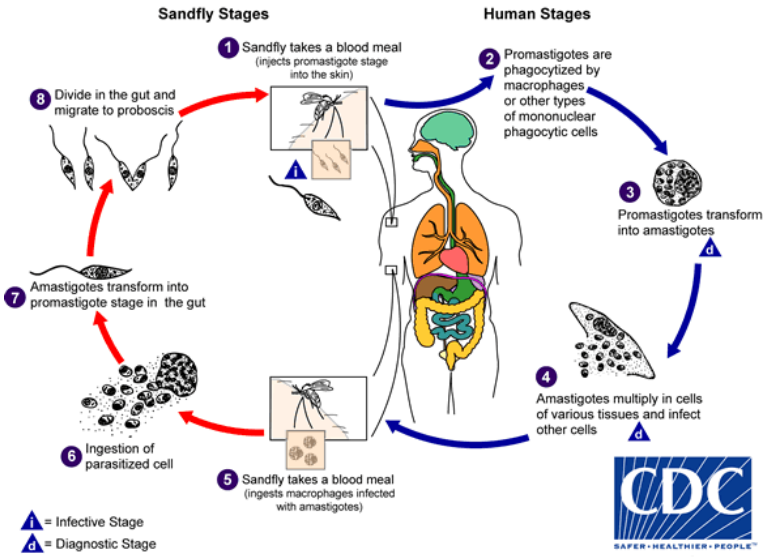


Figure 2: Biological cycle of *Leishmania spp*. Source: CDC.

2.1.3. Clinical presentations of leishmaniases

Clinical presentations of these diseases depend upon both the parasite species and the host's immune response. CL can be asymptomatic or with skin lesions. At first, a slight erythema can be observed at the sand fly bite site that can evolve to a local form of the disease characterized by a single self-resolving, sometimes ulcerated, skin lesion, or to a diffuse form of the disease with multiple non-ulcerative nodules (Reithinger et al. 2007). MCL involves a destructive mucosal inflammation that never heals spontaneously and leads to facial disfiguration (Reithinger et al. 2007). Life-long lesion scars arising from these diseases give rise to social stigma —especially for women, who are rejected for marriage and are separated from children in some cultures— as well as psychological suffering (Yanik et al. 2004). VL patients usually experience weight loss, irregular bouts of fever, and anemia accompanied by liver and spleen enlargement (World health Organization 2016). In some cases after its apparent complete resolution, VL evolves into a cutaneous form, known as post-kala-azar dermal leishmaniasis (PKDL) characterized by a self-healing macular, maculopapular, and nodular rash in a period of time that ranges from months to several years after suffering from VL (Zijlstra et al. 2003). Asymptomatic infections have also been described, affecting up to 70 % of total population of some highly endemic areas (Michel et al. 2011).

Due to its importance on global health, this work has focused on *Leishmania* species causing VL.

2.2 American Trypanosomiasis

American trypanosomiasis, also known as Chagas disease is a parasitic disease that affects more than 6 million people worldwide, with the endemic countries of South America being mostly infected (2017). The distribution of this disease principally occurs in poor and rural areas of continental Central and South America where the vector resides (World Health Organization 2015b; 2017). However, it is not uncommon to find human cases in non-endemic countries due to migrations, becoming therefore an emergent disease in areas such as Japan, USA, and Europe (Gascon et al. 2010).

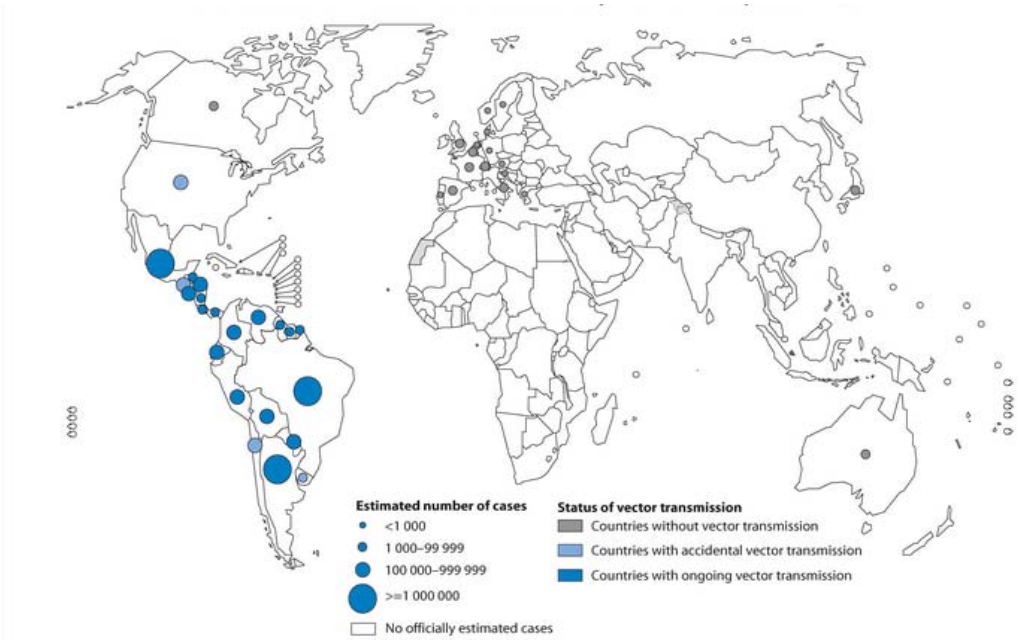


Figure 3: Distribution of cases of *T. cruzi* infection, based on official estimates and status of vector transmission worldwide, 2006-2009. Source: WHO.

2.2.1 Etiology and transmission of Chagas disease

Table 2: Scientific classification of *T. cruzi* parasites

AMERICAN TRYPANOSOMIASIS	
Kingdom	Protista
Phylum	Euglenozoa
Class	kinetoplastida
Order	Trypanosomatida
Family	Trypanosomatidae
Genus	*Trypanosoma*
Species	*cruzi*

American trypanosomiasis is a parasitic zoonosis caused by the protozoan parasite *Trypanosoma cruzi*. The parasite can also be found in domestic and sylvatic mammals of endemic areas such as opossums, raccoons, bats, and even pets such as dogs or cats, acting as reservoirs for human infection.

It is spread mostly by hemipteran insects of the Triatominae sub-family (Reduviidae family). More than 100 species of triatomine insects can transmit the parasite, g the genera *Triatoma*, *Rhodnius*, and *Panstrongylus* being highlighted due to their implication in human infection. These insects, popularly known as kissing bugs, are easily found in rural areas close to leafy

vegetation where burrow and palm trees are common, also taking over in wall and roof cracks in rudimentary buildings (Fernandes and Andrews 2012; Coura 2013; World Health Organization 2015b).

T. cruzi can also be transmitted by blood and organ transplantation (Martín-Dávila et al. 2008; Singh and Sehgal 2010), vertical transmission (Muñoz et al. 2009; Carlier et al. 2015), laboratory accidents (Herwaldt 2001), and even by the ingestion of food or beverages contaminated with infected feces of vector bugs or the vector itself (Alarcón de Noya et al. 2010).

2.2.2 Biological cycle of *T. cruzi*

T. cruzi is transmitted to its vertebrate host mainly through the feces of an infected triatomine bug deposited on the host just after taking a blood meal. Once on the victim's skin, the metacyclic trypomastigote form of the parasite migrates from the vector's feces through lesions on the skin or intact mucosal membranes of its future host, and infects cells close to the site of inoculation, where are temporary harbored in parasitophorous vacuoles. Parasites evolve to intracellular amastigotes, replicate asexually, and differentiate into trypomastigotes. Then, the host's cell ruptures and trypomastigotes are released either to invade neighboring cells or to spread through the bloodstream into a wide variety of distant tissues. New triatomine bugs are infected when taking a blood meal on the infected host. Bloodstream trypomastigotes are then carried by the insect and, once in its midgut, they transform into epimastigotes and multiply. In the hindgut, parasites are transformed into infective metacyclic trypomastigotes ready to be excreted in the next blood meal of the invertebrate host to complete the cycle (Fernandes and Andrews 2012; Centers for Disease Control and Prevention (CDC) 2016).

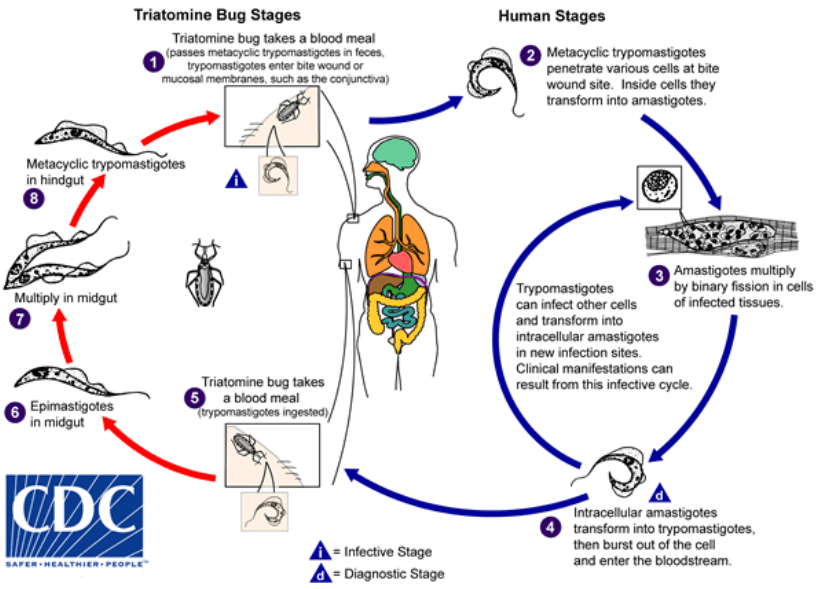


Figure 4: Biological cycle of *T. cruzi*. Source: CDC.

2.2.3 Clinical manifestations of Chagas disease

Once the parasite infects the vertebrate host, the acute phase of infection starts. This acute stage may last from some weeks to months and manifestations vary from asymptomatic patients to local inflammation at the inoculation site (swelling of a lid —Romaña's sign— or skin lesions), and unspecific symptoms such as fever, headaches, abdominal and chest pain, swelling of lymph nodes and tissues, and gastrointestinal disorders. Life-threatening myocarditis or meningoencephalitis can also occur during the acute phase —meningoencephalitis is the most frequent manifestation in HIV/AIDS co-infected patients—. This phase is accompanied by high parasitemia, which is reduced to sub-patent levels once acquired immunity is developed. Acute manifestations spontaneously subside in about 90 % of patients, even without trypanocidal drug treatment, leading to the chronic phase where patients remain mainly asymptomatic. About 30 % of chronically infected patients develop cardiac (chagasic cardiomyopathy), neurological, or gastrointestinal (colon or esophagus dilatation) disorders that could culminate in death up to 30 years after infection (Rodrigues Coura 2007; Fernandes and Andrews 2012; Nunes et al. 2013; Centers for Disease Control and Prevention (CDC) 2016).

2.3 Human African Trypanosomiasis

Human African trypanosomiasis, also known as sleeping sickness, is a vector-borne disease spread throughout 36 sub-Saharan African countries and mainly affecting remote rural populations. The vast majority of cases are reported in the Democratic Republic of Congo (World Health Organization 2017).

Despite 65 million people are still being at risk, the incidence of new cases in recent years has dropped due to control measures. Current estimations indicate that there are around 20,000 human cases of which less than 4,000 got infected in the last year. However, HAT is considered to have a 100 % fatality rate, and patients suffer from discrimination and are abandoned due to the neurological symptoms brought about by the disease (Brun et al. 2010; World Health Organization 2017).

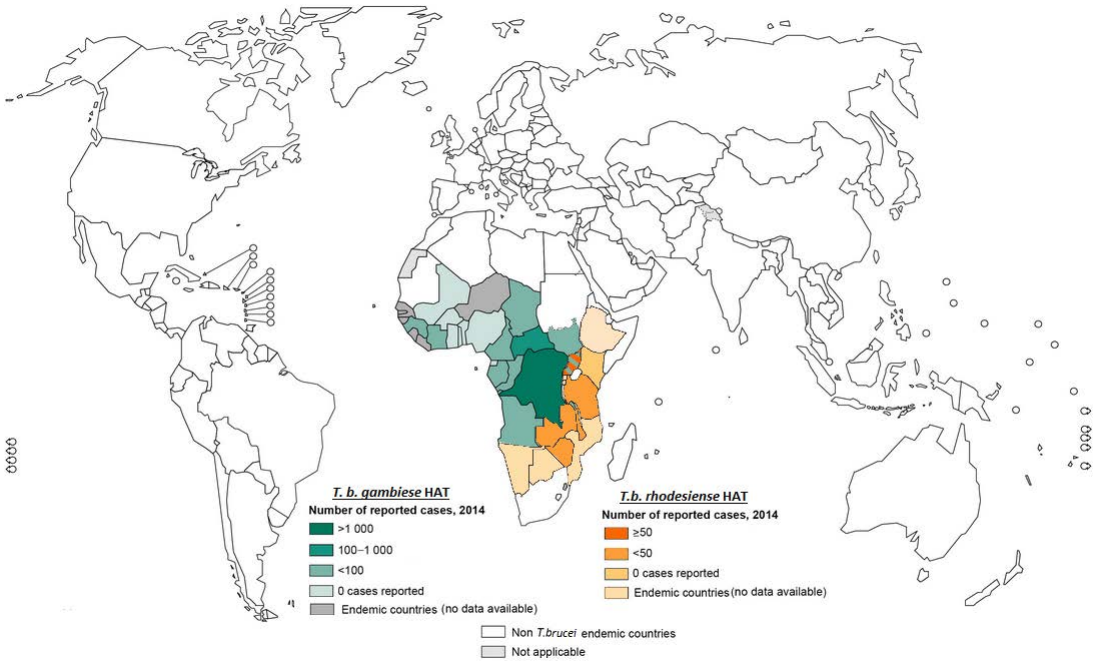


Figure 5: Distribution map of reported cases of *T. b. gambiense* and *T. b. rhodesiense* HAT in 2014. Modified from WHO.

2.3.1 Etiology and transmission of HAT

Table 3: Scientific classification of *T. brucei* parasites

HUMAN AFRICAN TRYPANOSOMIASIS	
Kingdom	Protista
Phylum	Euglenozoa
Class	kinetoplastida
Order	Trypanosomatida
Family	Trypanosomatidae
Genus	*Trypanosoma*
Species	*brucei*

Trypanosoma brucei is the protozoan parasite causing sleeping sickness. There are two different subspecies able to elicit the set of symptoms gathered under the name of sleeping sickness. *T. brucei gambiense* accounts for 98 % of HAT cases, and is spread in West and Central Africa, while *T. brucei rhodesiense* is considered to only accidentally infect humans and follows a southern and eastern distribution in the African continent (Brun et al. 2010). Species split apart by the Rift Valley and coexist only in Uganda, but it is expected to be overlapped in the future (Picozzi et al. 2005). *T. b. gambiense* and *T. b. rhodesiense* follow different transmission patterns. *T. b. gambiense* is considered an anthroponotic disease —particularly affecting dwellers from rural endemic areas— although the infection has also been reported to occur in animals with a complete cycle (Funk et al. 2013). Conversely, *T. b. rhodesiense* affects mainly livestock and wild animals, with cases due to this sub-species being incidentally and frequently reported in tourists from non-endemic areas (Neuberger et al. 2014).

T. brucei are mainly spread by the bite of tsetse flies (genus *Glossina*). Both male and female flies are hematophagous and can transmit the infection. There are 31 known species and sub-species of tsetse fly and all of them are potential vectors of trypanosomes, but *G. fuscipes*, *G. palpalis*, and *G. morsitans* are the ones mainly involved (Brun et al. 2010; Franco et al. 2014; World Health Organization 2017). Alternative reported transmission routes, include several cases of vertical transmission (Lindner et al. 2010), and laboratory accidents (Herwaldt 2001). Blood transfusions, organ transplantations (Martín-Dávila et al. 2008), and even sexual transmission (Rocha et al. 2004) may be other possible routes of transmission.

2.3.2 Biological cycle of *T. brucei*

The infection occurs when an infected tsetse fly injects metacyclic trypomastigotes of *T. brucei* in the subcutaneous tissue of a mammalian host. Due to the draining of the lymphatic system, parasites arrive in the bloodstream where they differentiate into asexually proliferating long slender trypomastigote forms. These bloodstream parasites are able to invade other body fluids and to penetrate the blood-brain barrier —reaching the cerebrospinal fluid, and thus, the central nervous system— and also the placenta. Some long slender parasites develop into non-replicative short stumpy forms which remain in the bloodstream and are ready to infect another tsetse fly when taking a blood meal. Within the insect's midgut, parasites evolve to procyclic trypomastigotes that migrate to the salivary glands of the vector, where they undergo multiplication, with only some of them acquiring the infective capacity as metacyclic trypomastigotes (Langousis and Hill 2014; Centers for Disease Control and Prevention (CDC) 2017a).

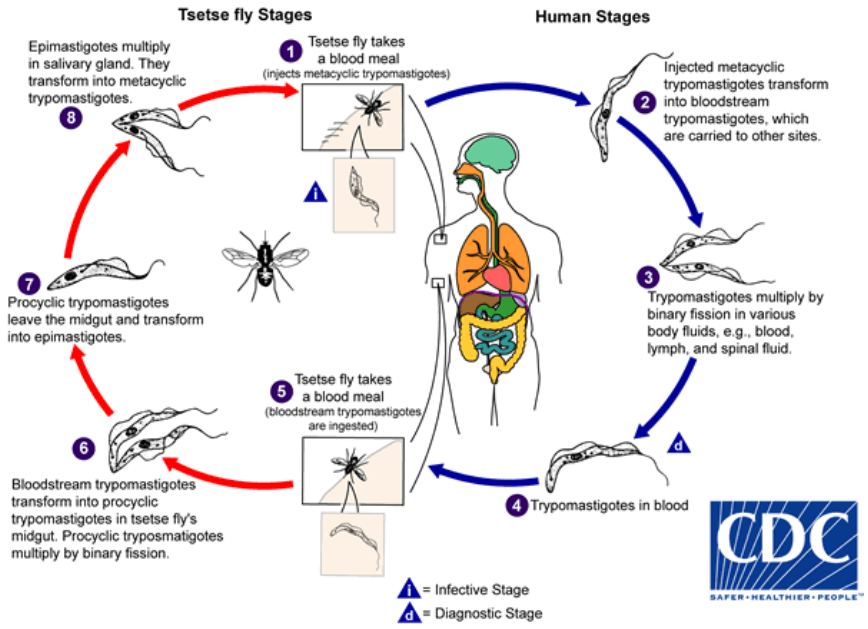


Figure 6: Representation of the biological cycle of *T. brucei*. Source: CDC

2.3.3 Clinical symptoms of HAT

The clinical presentation of HAT is similar for both *T. brucei* subspecies, but there are differences related the speed of disease progression and severity. Patients infected with *T. b.*

gambiense develop a chronic disease that can last for years without major signs of the disease. In contrast, *rhodesiense* sleeping sickness is more acute and leads to death in some months.

In a first stage, unspecific symptoms develop, such as headaches, bouts of fever, weakness, anemia, joint pains and pruritus, among others. Once parasites cross the blood-brain barrier, the nervous central system is affected, and neuropsychiatric clinical features appear with behavioral changes, loss of coordinated movements, and sleeping cycle disturbances (Franco et al. 2014; World Health Organization 2017). Although HAT has often been described as 100 % fatal if not treated, some reports point to the existence of asymptomatic subjects in endemic areas (Jamonneau et al. 2012).

2.4 Malaria

Malaria is considered endemic in most tropical and subtropical zones all over the world, threatening about 3.2 billion people that remain at risk. Only in the year 2015, the estimation of new malaria cases ranged between 149 to 303 million. However, this incidence implies a decline of 18 % over to the estimated 262 million for the year 2000. The vast majority of cases are recorded in sub-Saharan Africa, where the highest rates of mortality are also found —90 % out of a total of 438 000 deaths in 2015 — especially threatening the life of children under 5 years old (World Health Organization 2015a).

Malaria was considered endemic in Europe in the past, but its transmission declined during the 20th century mainly due to better socio-economic conditions (Zhao et al. 2016), with occasional outbreaks during World War I and II. Although the disease was officially declared eradicated from the European territory in 1975, malaria diagnosis is not unusual, with most of them being imported cases. Exceptionally, some cases of autochthonous malaria have been diagnosed with potential anopheline vectors involved (Santa-Olalla Peralta P; Vazquez-Torres MC; Latorre-Fandós E; Mairal-Claver P; Cortina-Solano P; Puy-Azón A; Adiego Sancho B; Leitmeyer K; Lucientes-Curdi and J; Sierra-Moros MJ 2010).

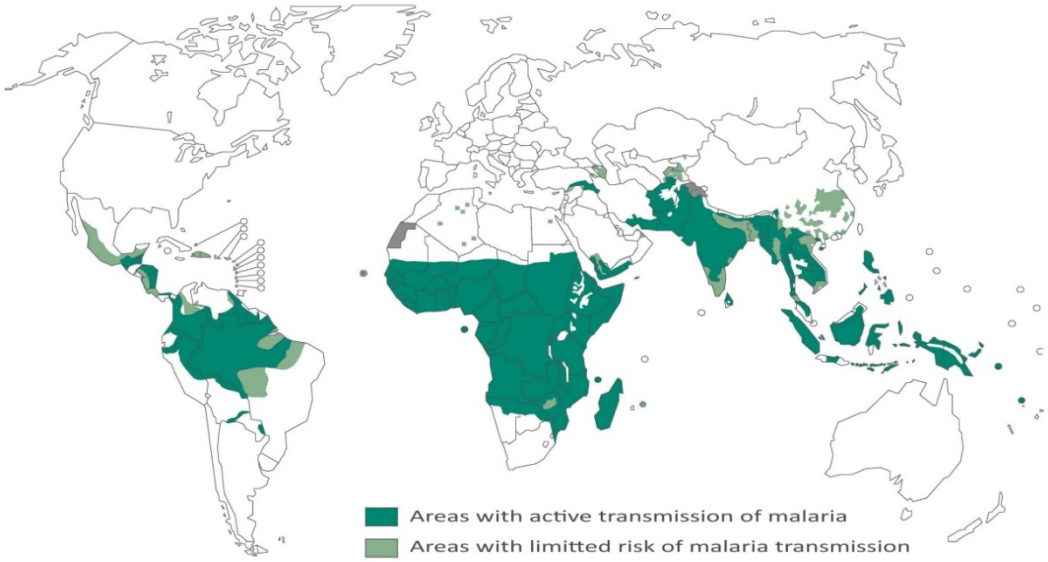


Figure 7: Areas of risk of transmission of malaria, 2010. Source: modified from WHO.

2.4.1 Etiology and transmission of malaria

Table 4: Scientific classification of *Plasmodium* parasites responsible for causing disease in humans.

PLASMODIUM	
Kingdom	Protista
Phylum	Apicomplexa
Class	Aconoidasida
Order	Haemosporida
Family	Plasmodiidae
Genus	*Plasmodium*
Species	*falciparum, vivax, ovale, malariae and knowlesi*

Malaria is caused by the protozoan parasite *Plasmodium.* There are more than 100 species of *Plasmodium* able to infect different living organisms. Four of them —*P. falciparum*, *P. ovale*, *P. malariae* and *P. vivax*— use humans as their natural hosts, causing disease following an anthroponotic transmission cycle (Centers for Disease Control and Prevention (CDC) 2017b).Many cases of zoonotic transmission of *P. knowlesi* malaria from macaques to humans have been reported in Southeast Asia, some of them with fatal outcome (Cox-Singh and Singh 2008). Although *P. falciparum* is responsible for most cases of severe malaria and mortality, *P. vivax* can be spread at lower temperatures, being the reason for its wider distribution.

These species of parasites are mainly transmitted by female mosquitoes belonging to the *Anopheles* genus. There are 30-40 species of *Anopheles* able to transmit the parasite,

depending on the geographical area, but the *Anopheles gambiae spp* and *Anopheles funestus* are especially efficient (Breman 2001).

Apart from the vectorial spread, other forms of transmission have also been described, mainly mediated by contact with contaminated blood as in blood transfusions (Singh and Sehgal 2010), sharing infected needles, antenatal infections due to transplacental transmission of infected erythrocytes to the fetus during pregnancy or at delivery (Malhotra et al. 2006; Poespoprodjo et al. 2010), and residual infected-erythrocytes in transplanted organs (Martín-Dávila et al. 2008). There are also reports of infections due to laboratory accidents (Herwaldt 2001).

2.4.2 Biological cycle of *Plasmodium*

Plasmodium has a complex biological cycle which has slight particularities depending on the parasite species. Overall, there are three differentiated phases: one so-called sporogonic phase, which takes place within the vector, and another two that develop in the human host. The first phase in humans is initiated when an infected female anopheles mosquito takes a blood meal, releasing sporozoites into the bloodstream, which are transported to the host's liver. Sporozoites then invade the hepatic cells, initiating the liver stage. There, they undergo multiplication producing thousands of haploid merozoites. In some plasmodium species —as *P. vivax*—, there is another parasite form known as a hypnozoite that persists in the liver and is responsible for future parasitemia relapses. Merozoites rupture the hepatic host cell and re-enter the bloodstream, infecting red blood cells and then initiating the erythrocytic cycle or blood stage of the parasite which is responsible for clinical symptomatology. Most of merozoites evolve to trophozoites when within the erythrocyte. Early trophozoites —generally referred as "ring stage", due to their uncanny resemblance with this object— mature into schizonts after several nuclear divisions without cytokinesis. When the schizont is mature, the host erythrocyte ruptures and new merozoites are released to the bloodstream parasitizing new red blood cells. Some merozoites differentiate into female or male gametocytes. During an anopheles mosquito blood meal, erythrocytes carrying gametocytes are picked up. The erythrocyte is digested, and gametocytes mature and merge, generating a diploid zygote that evolves into a motile ookinete that penetrates the mosquito midgut wall and develops into a sporozoite-filled oocyst. Sporozoites are released into the internal cavity of the mosquito from

where they migrate to the mosquito salivary glands, waiting there for the next blood meal to be injected (Centers for Disease Control and Prevention (CDC) 2017b).

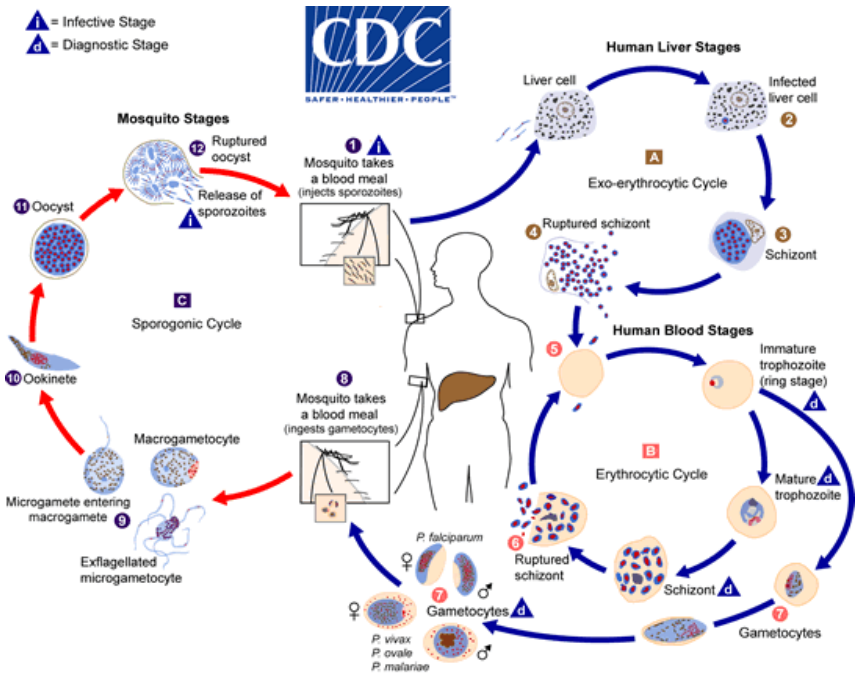


Figure 8: Biological cycle of *P. falciparum*. Source: CDC

2.4.3 Clinical symptoms of malaria

In general, the course of uncomplicated malaria includes acute fever and other unspecific symptoms such as lethargy, headache, chills, joint aches, abdominal discomfort and vomiting that appear within the first two weeks post-infection. If not properly treated during its early stages, the disease can worsen —specially in cases of *falciparum*-malaria— and evolve to potentially lethal malaria, where severe anemia, cerebral malaria, coma, hypoglycemia, metabolic acidosis and organ dysfunctions are frequent companions (Breman 2001; World Health Organization 2015a). Chronic infections with mild or no detectable symptoms, usually referred as “asymptomatic”, are frequent and grossly underestimated (Chen et al. 2016), reaching more than 30 % prevalence in highly endemic areas (Shekalaghe et al. 2007).

3. CONTROL OF THESE DISEASES

In recent years, despite the neglected condition of trypanosomiases, considerable progress has been made in the control of these diseases, mainly due to the integration of national and international surveillance and control initiatives (Moncayo and Silveira 2009; World Health

Organization 2015c). This fact, in addition to the improvement of socioeconomic and housing conditions, have been translated into a marked reduction of incidence and deaths by these parasitic infections (Moncayo and Silveira 2009; World Health Organization 2014). Current strategies for controlling these infections are based on the different approaches described below.

3.1 Vector and reservoir control

The vectorial transmission of these diseases turns vectors into principal targets for controlling parasite spread. The most common approaches in endemic areas are based on insecticide treated bed nets and indoor residual spraying. It is estimated that 70 % of new malaria cases expected between 2001 and 2015 were averted due to using these insecticide-based approaches (World Health Organization 2015a). Traps or insecticide-treated or toxic baits (Beier et al. 2012; Wamwiri and Changasi 2016) are other available options. However, vector resistance to insecticides — mainly pyrethroids — have been reported in several countries (Moncayo and Silveira 2009; Kawada et al. 2011; Hassan et al. 2012). For avoiding contact with triatomine bugs, housing improvement and good hygiene practices for food preparation are also required (Coura 2013).

Asymptomatically infected animals and humans play an important role in the persistence of the parasite in the environment. Therefore, screening of populations at higher risk of infection or disease in endemic areas —and also blood and organ donors— is a recommended procedure. However, as regards pregnant women, infants, and schoolchildren in malaria endemic areas and with seasonal transmission, rapid diagnostic tests have been reported to miss up to 50 % of asymptomatic infections due to low parasitemias (Chen et al. 2016). In the case of leishmaniasis, controlling canine leishmaniasis (CanL) has shown to be an effective tool for reducing the zoonotic spread of the disease (Palatnik-de-Sousa 2012). Preventive approaches such as vaccination of pet dogs and the use of insecticide-treated collars or spot-on insecticides, in addition to the treatment of infected animals, are common procedures in veterinarian medicine in Southern Europe (Podaliri Vulpiani et al. 2011; Otranto and Dantas-Torres 2013). To date, two vaccines have been commercialized for CanL in Brazil —albeit one of them recently had its marketing license withdrawn— and another two in Europe (Dantas-Torres 2006; Carcelén et al. 2009; Fernandes et al. 2012; Bongiorno et al. 2013). The culling of *Leishmania*-seropositive dogs is a current practice in Brazil but, besides posing an ethical

dilemma, it has shown low efficacy in reducing human incidence of the disease (Duarte Moreira et al. 2004; Grimaldi Jr. et al. 2012).

3.2 Chemotherapy

No vaccines have yet been commercialized for humans against these pathogens, so the main approach for their management is based on pharmacological treatment. Currently, there are some commercialized drugs to fight against these parasites that have achieved highly efficient results. However, the increasing number of drug-resistant and toxicity cases and the limited economic resources of the most frequently affected patients complicate the choice of available compounds. In addition, some evidence seem to connect HIV infection with relapses of these diseases after anti-parasitic therapies in immunocompromised patients (Burza et al. 2014), also increasing the risk of adverse drug reactions (Alvar et al. 2008).

3.2.1 Treatment of VL

There are many therapeutic options currently available for VL, but treatment efficacy depends on several factors related to the host immune system, the parasite species, the geographical area, and the socio-economic status. These compounds are not widely affordable and the development of resistances and adverse effects requires corrective measures and other pharmacological strategies that increase the price.

The WHO first line choice are the pentavalent antimonials (sodium stibogluconate and meglumine antimoniate). However, these compounds can cause severe adverse reactions including cardiotoxicity, pancreatitis (Gasser et al. 1994), nephrotoxicity, mutagenic effects (Lima et al. 2010), and death. In addition, these compounds require a long course (up to 1 month) of parenteral administration with hospitalization and, although they reach between 80-90 % therapeutic rates, resistances are frequent (Alvar et al. 2006; Barrett and Croft 2012). Amphotericin B is the first therapeutic option in areas where pentavalent antimonials are nearly obsolete due to the high resistances rates —as is the case in Bihar, India— (Sundar 2001). Adverse events described for this treatment vary from fever and chills to potentially fatal hypokalemia, anaphylaxis, and nephrotoxicity. Liposomal formulations of this compound (Ambisome) reduce the frequency of adverse reactions, but also increase its price; thus limiting its use to wealthy areas. Amphotericin formulations reach up to 98 % efficacy rates but

require intravenous infusion and health care assistance. Miltefosine constitutes a validated oral therapy to VL, with efficacy rates ranging between 80-90 %. However, long administration periods (up to 1 month) are needed, and teratogenic and severe gastrointestinal effects have been described in addition to high rates of relapse and resistances (Sundar and Olliaro 2007). Paromomycin was the last licensed compound for anti-leishmanial purposes. This drug shows variable efficacy depending on the species and ecozone, ranging from 50 % up to 90 %, and among the adverse effects reported, the most common are ototoxicity and impaired liver function (Sundar et al. 2007).

In veterinary medicine, CanL treatment relies on pentavalent antimonials or miltefosine often in combination with allopurinol. These last compound must be co-administered with the other drugs for the first month and then continued alone (Solano-Gallego et al. 2011). Using the same therapeutic compounds in CanL and in VL —pentavalent antimonials and miltefosine—, raises the risk of appearance of new resistances as CanL therapies do not usually achieve complete clearance of the parasite (Gramiccia et al. 1992). Despite allopurinol being rarely used in human VL therapies and therefore constitutes a good option for CanL treatment, resistances to this compound exist and are responsible for CanL relapses after treatment (Yasur-Landau et al. 2016), in addition to its low efficacy when used as monotherapy (Koutinas et al. 2001).

3.2.2 Treatment of Chagas disease

In the case of Chagas disease, anti-parasitic treatment aimed against *T. cruzi* is recommended in case of acute infection, in reactivation of the disease due to immunosuppression, in children, and in cases of congenital infection. Currently, there are two marketed drugs: benznidazole (BZD) and nifurtimox (NFX). The efficacy of these drugs is better the sooner the treatment starts after infection, inducing complete resolution of the disease in 70 % of patients in the acute phase, and reaching almost 100 % efficacy in congenitally infected patients (Guedes et al. 2011; Nunes et al. 2013). However, full treatment requires long periods of administration —up to 60 days— and three doses per day (Apt 2010). Current approved treatments show reduced efficacy in chronic stage patients, and therefore their use is controversial due to frequent adverse reactions, but BZD treatment has been reported to slow down the progression of the chronic stage of the disease (Viotti et al. 2006). BZD and NFX are inadvisable during pregnancy, breast-feeding mothers, and patients with kidney or hepatic

dysfunctions. the main problems are the appearance of resistant parasite strains and relative high frequency of adverse reactions —up to 40 % of treated patients— (2017). The most common adverse reactions are gastrointestinal and psychiatric disorders in the case of NFX, and mild to severe skin reactions and hematological alterations with depression of bone marrow when BZD is administered. In addition, some studies report a tumorigenic effect for both compounds (Castro et al. 2006).

3.2.3 Treatment of HAT

The first choice drug for treating HAT depends on the stage of the disease and on the parasite sub-species.

In early stages of the disease, pentamidine and suramin are recommended as first line compounds for *T. b. gambiense* and *T. b. rhodesiense*, respectively. These compounds can cause low to mild transient adverse events, and in the case of suramin, hypersensitivity reactions, peripheral neuropathies and renal problems have also been described (Brun et al. 2010).

In the second stage of the disease —when the central nervous system is affected—, treatment is more complicated and aggressive, as drugs must cross the blood brain barrier, thus increasing toxicity. Melarsoprol, an arsenic derived compound is currently recommended as a first-line drug against second-stage *T. b. rhodesiense* . This compound is also effective against *T. b. gambiense*, but its high toxicity, the emergence of resistances, and the availability of other effective compounds makes it a second-line choice for this species. Adverse reactions occur frequently, and can sometimes be life-threatening. Encephalopathy syndrome is the most dangerous, occurring in less than 10 % of patients and with a mortality rate that can exceed 50 % (Pépin et al. 1995). Skin and neuropathological processes have also been related to this drug. Curiously enough, the melarsoprol mechanism of action still remains unknown, but seems to target thiol containing enzymes of the parasites such as GAPDH, which is relevant in this work (Nok 2003). Eflornithine is effective only for gambiense HAT and although it has less adverse effects, blood alterations —due to bone marrow toxicity— (Milord et al. 1992), gastrointestinal problems, and even seizures are not uncommon. In addition, due to its short life, it requires intravenous infusion four times a day for two weeks, making this treatment unaffordable for most people living in rural areas. In recent years, the combination of this

compound with nifurtimox —previously described as effective treatment for American trypanosomiasis— is considered as first-line treatment for second stage gambiense HAT (Priotto et al. 2009). The combination of these compounds reduced severe adverse effects and increased the cure rate from 92 % (eflornithine alone) to 96 % (Reviewed in depth by other authors (Jacobs et al. 2011; Babokhov et al. 2013)).

3.2.4 Treatment of malaria

Malaria treatment depends on the evolution of the disease, the parasite species, and resistances in the area of prescription. There are many compounds available for combating malaria, but drug resistance is a subject of concern. At first, uncomplicated malaria cases were treated with chloroquine (CQ) monotherapies, both for *P. vivax* and *P. falciparum* malarias. Unfortunately, resistances quickly appeared and this regime was then replaced with sulfadoxine-pyrimethamine treatments, which in turn were replaced by the artemisinin-based combination therapies (ACTs) recommended by the WHO. CQ is still currently considered as a first line compound for *P.vivax* malaria if there are no resistances to this drug in the area, otherwise ACTs are prescribed. ACTs are the most effective medicines available and show less than 5 % treatment failure rates (World Health Organization 2015d). This strategy allows combining different long-acting compounds —lumefantrine, piperaquine, amodiaquine, mefloquine, or sulfadoxine-pyrimethamine— with short-acting artemisinin derivatives —artemether, artesunate, or dihydroartemisinin—, achieving the killing of susceptible parasites to artemisinin during the first days and eliminating the remaining ones later, thus avoiding relapses. This therapy also confers a post-treatment temporary prophylactic effect, which prevents new infections while drug concentrations in blood exceed the minimum inhibitory concentration for the parasite (Nosten and White 2007). Nevertheless, resistances to different compounds —artemisinin derivatives included— and even multidrug resistances have been found (World Health Organization 2015d), and ACTs price is high when compared to other single compounds. Artemisinin derivatives monotherapy must be avoided, as it has been shown to promote the emergence of resistances. Primaquine treatments are recommended in addition to other therapies to avoid relapses. Primaquine should not be given to pregnant women, and a screening test for glucose-6-phosphate dehydrogenase deficiency (G6PD) must be performed prior to administration due to severe complications causing red cell rupture and death (World Health Organization 2015d). Reviewed in depth by other authors (D'Alessandro 2009; van Vugt et al. 2011).

Severe malaria must be immediately treated with a full course of artemisinin derivatives (preferentially artesunate) —either parenteral —or rectal, if not tolerated—or cinchona alkaloids (quinine or quinidine), followed by a complete ACT protocol.

Intermittent presumptive treatment is a strategy mainly used as a prophylactic that consists of giving effective doses of antimalarial drugs at defined intervals to highly susceptible populations such as pregnant women during their second and third trimester of pregnancy, and children in endemic areas in order to maintain therapeutic drug levels in blood during higher risk periods (White 2005). The most used compounds for this strategy are sulfadoxine-pyrimethamine, with additional amodiaquine in the case of seasonal treatments (World Health Organization 2015d).

3.3 Vaccination

As previously described, there are several effective drugs to fight against the infection, which have achieved highly efficient results. But due to wild life cycles of parasite transmission —which turns the parasite spread control into a more complex task—, the increasing number of drug-resistant and toxicity cases, as well as the limited access to medicines and preventive tools in the lowest socioeconomic environments where these parasites are principally spread, there is a special need for developing a preventive vaccine.

To date, no vaccine against these parasitic infections has been approved for human use. Major obstacles to developing efficient vaccines for these diseases are related to the incomplete knowledge of the characteristics of a protective immune response. During the evolutionary process, the adaptation to live within the host endowed these parasites with sophisticated immune evasion strategies, such as antigenic variation, depletion of immune cells involved in protection, general immunosuppression, complement evasion, and infected-cell sequestration, to avoid entering into the spleen for elimination (Gupta et al. 2013; Cardoso et al. 2015; Dinko and Pradel 2016; Stijlemans et al. 2016). This fact, in addition to the lack of faithful experimental models, has hampered the development of efficient vaccines (Magez et al. 2010; Tabel et al. 2013; Srivastava et al. 2016). Despite this, countless attempts were carried out with different vaccination experimental approaches.

The vast majority of vaccines available against microorganisms can be grouped into 3 categories depending on their composition: live, attenuated, or killed organisms; protein vaccines; and DNA based strategies. The latest technologies have enabled their effect to be potentiated by accompanying the initial vaccines with novel adjuvants, recombinant viral vectors or proteins, or following heterologous prime-boost regimes. In the case of the studied protozoan diseases, all these different strategies have been tested with variable results in rodent models. However, just some of them have made it to move on to further research on human models.

Vaccine strategies for Chagas disease rely on the search of a strong cellular immune response with the activation of CD8+ cytotoxic T cells (Quijano-Hernandez and Dumonteil 2011). Recombinant parasite proteins, the use of viral vectors, DNA vaccines, and heterologous prime-boost strategies have demonstrated to protect (or partially protect) and increase survival of *T. cruzi*-challenged mice (Quijano-Hernandez and Dumonteil 2011; Prepared for WHO PD-VAC 2014a; Rodríguez-Morales et al. 2015). In contrast, desirable immunity against *T. brucei* involves memory B cell creation and long lasting antibody production (Magez et al. 2010). For that purpose, variable and non-variable antigens of the parasite in combination with adjuvants or expressed in viral vectors have been assayed. These strategies achieved protection or partial protection in mice models (Magez et al. 2010; La Greca and Magez 2011). Despite this, all vaccine candidates for African and American trypanosomiasis are still in the preclinical phase of testing, far from clinical trials (Magez et al. 2010; Prepared for WHO PD-VAC 2014a; Beaumier et al. 2016).

Three vaccine candidates for malaria prevention are currently in an advanced stage of development. Recently, the *P. falciparum* malaria vaccine RTS,S/AS01, based on the *P. falciparum* CS protein, gained the positive scientific opinion of the European Medicines Agency. The WHO Strategic Advisory Group of Experts on Immunization (SAGE) and the Malaria Policy Advisory Committee (MPAC) recommended its pilot implementation, thereby possibly becoming the first malaria vaccine in the field (World Health Organization 2015a) and the only phase III malaria vaccine candidate (Riley and Stewart 2013). Different approaches with other two malaria vaccine candidates are currently in phase II of development: the ChAd63/MVA ME-TRAP, based on recombinant virus technology (Chimpanzee Adenovirus 63 Modified Vaccinia Ankara Multiple epitope thrombospondin adhesion protein (ME-TRAP))

(Afolabi et al. 2016), and the PfSPZ, which contains radiation attenuated sporozoites (Seder et al. 2013) (http://www.who.int/immunization/research/development/Rainbow_tables/en/).

Several *Leishmania* vaccines for human use have been assayed in clinical trials, but there is still long way to go to see them marketed. There are currently two promising vaccine candidates in development for human VL. Both are recombinant protein vaccines composed by the *Leishmania* antigen LeishF3 (NH-SMT) formulated with the adjuvant glucopyranosyl lipid A (GLA-SE) (Coler et al. 2015). The second recombinant vaccine, MuLeVaClin, also carries the KMPII recombinant antigen and a recombinant salivary protein of the sand fly vector (European Commission 2015). The LEISH-F3+GLA-SE vaccine safety and immunogenicity has already been tested in healthy volunteer adults from a non-endemic region (Washington), and a second phase I will take place in the endemic region of Bangladesh (Prepared for WHO PD-VAC 2014b; Coler et al. 2015). In the case of the MuLeVaClin vaccine, preliminary results showed that the formulation does not provide strong protection against an intravenous challenge with *L. infantum* promastigotes in mice, and is now being tested on the hamster model (European Commission 2015). There is currently another vaccine candidate formulated for patients with persistent PKDL (ChAd63-KH), which is already in phase II trials, and still recruiting volunteers in Sudan. In this case, it is a therapeutic vaccine (ClinicalTrials.gov 2016).

In addition, not all the research has been focused on developing protective vaccines based on potentiating a desirable immune response against the parasite in the host. Some trials have been addressed to create vaccines to block parasite transmission from vectors to hosts, and also vaccines against disease complications (Stijlemans et al. 2007)(Magez et al. 2010; Hill 2011; Rezvan and Moafi 2015).

4. NEW APPROACHES TO MANAGE THE SPREAD AND DISEASES ARISING FROM THESE PARASITOSES

The WHO's work on these tropical diseases promotes research for developing safe and affordable medicines, diagnostic tools, and vaccines. Our choice was drug repurposing and immunity potentiation in a vaccine trial.

4.1 Drug repurposing

The increasing number of drug-resistances to current treatments, toxicity, and the limited access to medicines in the lowest socio-economic environments where tropical parasitic diseases are mainly spread, make it urgently necessary to develop new affordable therapies and efficient vaccines.

The areas most affected by tropical arthropod-borne parasitic infections are the developing countries, thus new therapies should be easy to administer and for short periods, preferably resistant to harsh conditions, and affordable for residents in endemic areas. Globally, only a small percentage of total investment in health research is destined to drug discovery for tropical diseases (Pink et al. 2005). In addition, developing new drugs involves large budgets and long lead-times. In this setting, drug repositioning or repurposing is a fast and cheaper alternative because it employs validated molecular targets and drugs for new indications. This is especially helpful for neglected diseases, where large investments are almost an utopia (Nwaka et al. 2009; Andrews et al. 2014; Jin and Wong 2015).

Trypanosomatidae, Plasmodiidae, and many other organisms of the clades formerly grouped in the protist kingdom, exhibit significant differences compared to what were considered paradigms of the eukarya domain that can be used as therapeutic targets (Simonite 2005). In this study, we are going to focus on parasite energy obtaining strategies, based on the principle that these organism share similar metabolic features with cancer cells.

4.1.1 Energy obtaining metabolism in eukaryotic cells

Every cell needs a source of energy to maintain homeostasis. One of the most important molecules to support energy and biomass generation is glucose. Glycolysis is a highly conserved pathway among living organisms which consists of the catabolic metabolism of glucose to produce ATP and pyruvate. Classic glycolysis –termed Embden-Meyerhof-Parnas pathway— generally takes place in the cytosol and consists of two phases. The first phase of this pathway is known as the preparatory phase —as ATP is invested— and starts with the phosphorylation of one molecule of glucose to glucose-6-phosphate (G6P) investing one molecule of ATP. In the next step, G6P is isomerized to fructose-6-phosphate, which in turn is phosphorylated to fructose 1,6-biphosphate (F1,6BP) in a step where another molecule of ATP

is consumed. F1,6BP is split into two three-carbon sugar phosphates known as glyceraldehyde 3-phosphate (G3P) and dihydroxyacetone (DHA), which can be interconverted. At this point, the second glycolytic phase starts, known as the payoff phase —as ATP is yielded— where all steps are duplicated, as from one molecule of glucose, two molecules of G3P can be obtained. The first step of this second glycolytic phase starts with G3P being oxidized —where NAD+ reduction to NADH is needed— and phosphorylated forming 1,3-biphosphoglycerate (1,3BPG), which in turn is transformed to 3-phosphoglycerate (3PG) in a process that yields a molecule of ATP. 3PG is then rearranged into 2-phosphoglycerate, which is subsequently dehydrated to produce phosphoenolpyruvate. This metabolite is then transformed into pyruvate, yielding a new molecule of ATP (Figure 9). To summarize, from one glucose and two ATP molecules, two molecules of pyruvate are obtained in addition to 4 molecules of ATP and 2 of NADH. Thus, the net energy production is two molecules of ATP.

Classically, in the presence of oxygen, pyruvate is led to the mitochondrion and oxidized to acetyl-coenzyme A, which enters the tricarboxylic acid (TCA) cycle —also termed the Krebs cycle or citric acid cycle— in the mitochondrial matrix where it is degraded to CO_2, producing high amount of ATP when coupled to oxidative phosphorylation (OXPHOS) in the inner mitochondrial membrane (Figure 9). On the other hand, when oxygen is not available, pyruvate is reduced to lactate and transported out of the cell.

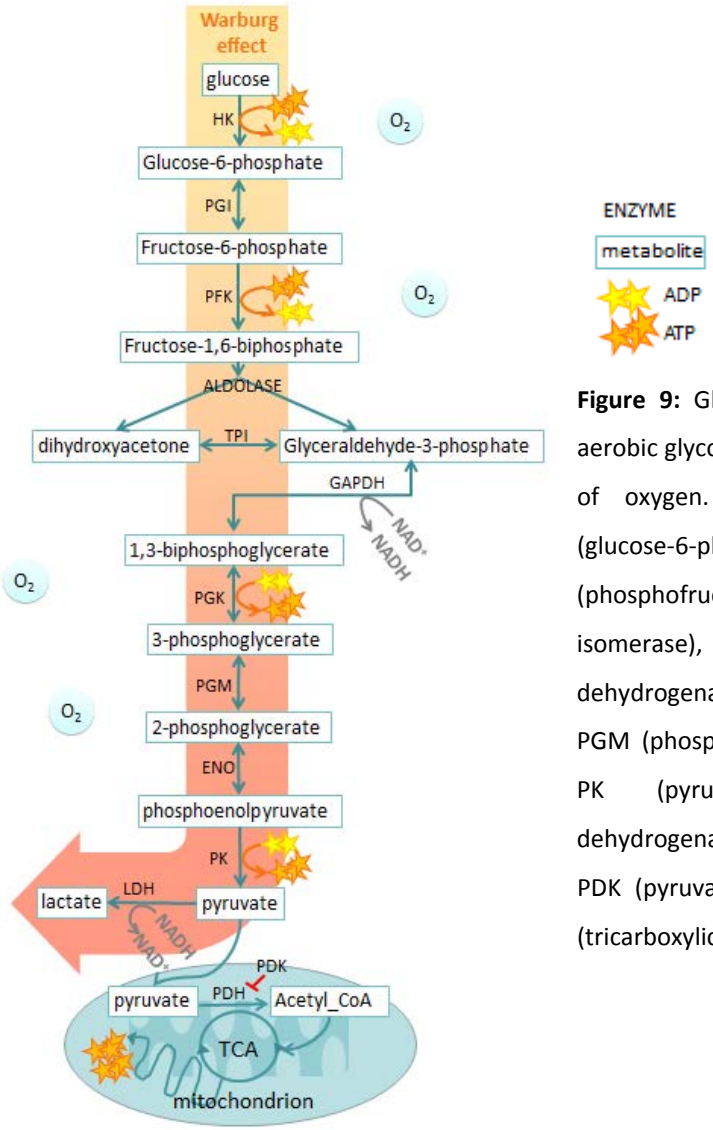


Figure 9: Glycolytic flux in eukaryotic cells and aerobic glycolysis (Warburg effect) in the presence of oxygen. Enzymes: HK (Hexokinase), PGI (glucose-6-phosphate isomerase), PFK (phosphofructokinase), TPI (triose-phosphate isomerase), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), PGK (phosphoglycerate kinase), PGM (phosphoglycerate mutase), ENO (enolase), PK (pyruvate kinase), LDH (lactate dehydrogenase), PDH (pyruvate dehydrogenase), PDK (pyruvate dehydrogenase kinase), and TCA (tricarboxylic acid cycle).

Most cancer cell types are known to have a modified glycolytic metabolism, aerobic glycolysis, consisting of glucose transformation to lactate, even in the presence of oxygen. This process is known as the "Warburg effect" and allows cancer cells to survive and multiply faster than regular cells (Warburg O, Posener K 1924; Warburg 1956). This glucose metabolism is also found in other cell types, and is considered a common feature of highly metabolically active cells, immune system activated cells, and proliferation committed cells. At first, it was hypothesized that tumor transformation was a result of a mitochondrial defect potentiating this aerobic glycolytic metabolism, but current knowledge points to that cancer cells do not necessarily have impaired mitochondrion but a reduced flux to it with respect to glycolysis and is highly involved in anabolic processes (Vander Heiden et al. 2009) (Figure 9). Aerobic glycolysis involves increased glucose requirements, as every molecule of glucose yields the net production of two molecules of ATP, which is a significant lower amount of energy than that

provided by glycolysis coupled to OXPHOS (36 ATP in total). Nevertheless, aerobic glycolysis perfectly meets highly active cells metabolic requirements, as the rate of ATP production is faster than with OXPHOS due to the enhanced glucose catabolism. In addition, the increased amount of glycolytic intermediates is destined to meet several biosynthetic needs of these highly replicating cells, such as the synthesis of lipids, several amino acids —serine, glycine and cysteine, which are, in turn, basic molecules for folate and methionine cycles (Locasale 2013)—, nucleotides, and the recovery of the reducing agent NADH —via the Pentose Phosphate Pathway (PPP)—(Vander Heiden et al. 2009). Reviewed in depth by other authors (Lunt and Vander Heiden 2011; Yu et al. 2016).

The mTOR pathway could also be a main therapeutic target for proliferation-committed cells. The mechanistic target of rapamycin (mTOR) configures a highly conserved route among eukaryotic organisms that orchestrates cell adaptations to environmental changes by controlling at transcriptional and translational level, cell survival strategies, cell proliferation, and metabolic adaptations regulating the balance between catabolism and anabolism according to cellular needs (Singh et al. 1979; Sabatini. Laplante. 2013). All these effects are mediated by two different complexes in mammalian cells where mTOR complex 1 (mTORC1) mediates cellular processes related to autophagy, translation, ribosome biogenesis, and transcription, whereas mTOR complex 2 (mTORC2) controls actin cytoskeleton organization and cell survival strategies. mTOR acts as a key homeostatic sensor that integrates micro-environmental and proliferation signals with bioenergetic metabolism to regulate biomass production and cell survival (van Dam et al. 2011; Sabatini. Laplante. 2013), and it is not uncommon that cancer cells present with a dysregulated PI3K/AKt/mTOR. It is also noteworthy that mTOR is strongly related to glycolysis, being able to induce the expression of glucose transporters and many of the enzymes that belong to this pathway —PKM2, PDK1, HKII, PFK2, PFK1—, partly by promoting hypoxia induced factor 1 (HIF-1) activity, and also through the direct activity of its upstream protein Akt (Semenza et al. 1994; Düvel et al. 2011). In addition, mTOR conforms a regulative link between cell metabolism and innate immunity, where the activation of Antigen Presenting Cells (APCs) through Toll like receptors (TLRs) entails a metabolic reprograming from oxidative phosphorylation to aerobic glycolysis (reviewed by Weichhart et al. (Weichhart et al. 2015)).

4.1.2 Energy obtaining metabolism in highly replicative asexual stages of Trypanosomatids and *Plasmodium* within the host

As a result of their changing life conditions, these pathogens exhibit different metabolic profiles during their biological cycle. Of special interest is the energetic metabolism these parasites present during their stage associated with pathology within the host, where these parasites undergo rapid multiplication.

In intraerythrocytic *Plasmodium* stages, as happens in the previously referred eukaryotic cells, the whole glycolytic process takes place in the cytosol. In contrast, in trypanosomatids the first 7 enzymes of the glycolytic pathway are located in a unique peroxisome-like organelle called glycosome, where the first steps of glycolysis take place, and is followed by last reactions occurring in the cytosol of the parasite (Bringaud et al. 2006; Opperdoes and Szikora 2006). Energy metabolism in trypanosomatids is not fully understood and it is only partially demonstrated (Bringaud et al. 2006; Opperdoes and Coombs 2007; Tielens and van Hellemond 2009; Subramanian et al. 2015). However, metabolic pathways reconstruction from genome-based information and evidence from informatics models led us to the current knowledge. To date, it is known that the bloodstream stages of *T. brucei* show the most simple metabolism of trypanosomatids, where the sole source of carbons for ATP production is glucose via glycolysis, and pyruvate is the main end product (Coley et al. 2011) (Figure 10). Maintenance of the redox balance in bloodstream *T. brucei* stages depends on a mitochondrial electron transport chain that does not have cytochrome containing complexes, but instead contains an alternative oxidase known as glycerol-3-phosphate oxidase (Chaudhuri et al. 1998) that is connected to the DHAP/glycerol-3-phosphate shuttle, and therefore to glycerol production (Figure 10). This electron transport does not involve transmembrane proton translocation, and is therefore not linked to ATP production. *T. cruzi* and *Leishmania spp* can degrade other metabolites such as amino acids, and glycolysis is extended to a reversible succinate production pathway within the glycosome (Besteiro et al. 2002; Maugeri, Cannata, and J. Cazzulo 2011). In addition, the resulting pyruvate is transformed into alanine or transported to the mitochondrion, instead of being excreted to the external milieu (Figure 10). These parasites show a much more complex mitochondrial metabolism that is strongly connected to anabolic processes and OXPHOS, with a cytochrome containing respiratory chain (Tielens and van Hellemond 2009). Notwithstanding, the high metabolic demand to support rapid multiplication in the mammalian host turns glycolysis into an essential process for these amastigote-stage

trypanosomatids (Verlinde et al. 2001; Subramanian et al. 2015) with special interest for NADPH regeneration and for the correct functioning of their PPP (Maugeri et al. 2003; Maugeri, Cannata, and J.-J. Cazzulo 2011). Like that of trypanosomatids, *P. falciparum* intraerythrocytic asexual forms are highly dependent on glucose consumption, and preliminary results indicate these parasites are aerobic glycolytic organisms with a modified TCA mainly fueled by glutamine and glutamate and low OXPHOS (Salcedo-Sora et al. 2014) that contains an alternative complex I as NADH dehydrogenase (Biagini et al. 2006). However, some studies indicate the TCA in *P. falciparum* trophozoites may not be directly connected to glycolysis, as the pyruvate dehydrogenase complex is located in the apicoplast of the parasite (Olszewski et al. 2010). Same as *T. brucei* bloodstream stage, *P. falciparum* glycolysis products are pyruvate and alanine. However, *P. falciparum* has a functional lactate dehydrogenase which transforms part of the generated pyruvate into lactate that is also excreted to the external milieu (Salcedo-Sora et al. 2014; Penkler et al. 2015) (Figure 10).

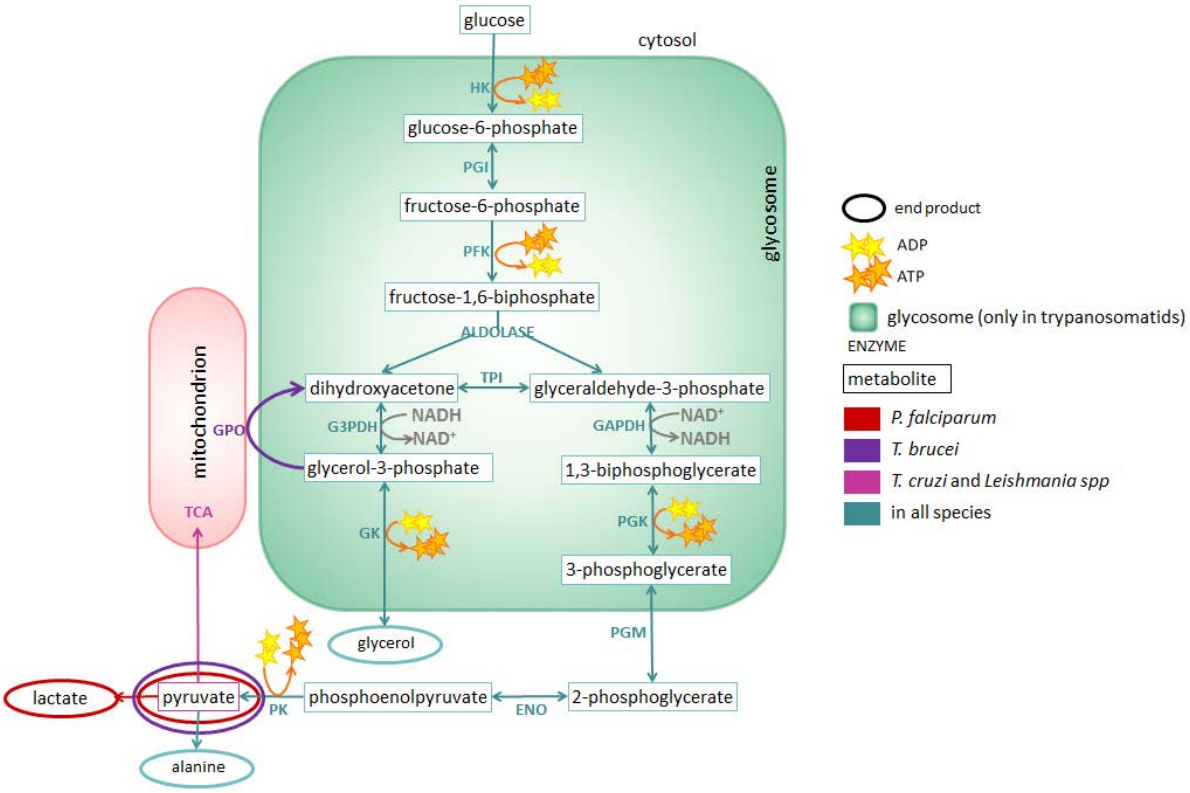


Figure 10: Unified and simplified glycolysis of bloodstream *T. brucei*, *T. cruzi* and *Leishmania* amastigotes, and *P. falciparum* trophozoites. Acronyms: HK (hexokinase), PGI (glucose-6-phosphate isomerase), PFK (phosphofructokinase), TPI (triose-phosphate isomerase), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), PGK (phosphoglycerate kinase), PGM (phosphoglycerate mutase), ENO (enolase), PK (pyruvate kinase), GPO (glycerol-3-phosphate oxidase), G3PDH (glycerol-3-phosphate dehydrogenase), GK (glycerol kinase), and TCA (tricarboxylic acid cycle).

As a result, glycolysis appears to be a particularly suitable target to be blocked, as these parasites tend to be more dependent on this pathway than the quiescent cells from differentiated tissues of their hosts (Renslo and McKerrow 2006).

4.1.3 Energy metabolism as a target for protozoan parasite killing purposes

The attractiveness of these bioenergetics pathways has not escaped avid authors and the literature have established these pathways as probable and interesting targets for protozoan diseases (Verlinde et al. 2001; Jacobs et al. 2011; Saldivia et al. 2013). Likewise, some energy modulators are already in use in cancer diagnosis, or in advanced phases of clinical trials (Scatena et al. 2008; Granchi and Minutolo 2013), thus facilitating repurposing.

In this study, the following compounds were selected for parasite killing purposes: 3-bromopyruvic acid (3BP), 2-deoxy-D-glucose (2DG), lonidamine (LND), dichloroacetic acid (DCA), metformin (MET), and sirolimus (SIR) (table 5).

2DG, LND, and 3BP block early stages of the glycolysis pathway, as they directly and indirectly interact with the first step of glycolysis (Paggi et al. 1988; Kurtoglu et al. 2007). This step is mediated by hexokinase (HK) and orchestrates the phosphorylation of glucose to G6P.

2-deoxy-d-glucose (2DG) has been used as a substrate to measure glucose intake in many cell systems due to its condition as a mannose analog, and also in positron emission tomography PET scanning for cancer diagnostic purposes. It blocks glycolysis because it cannot be further metabolized after phosphorylation by HK (Nirenberg and Hogg 1958).

Lonidamine (LND) inhibits mitochondrial-binding HK1 in tumor cells (Paggi et al. 1988), thus blocking glycolysis and damaging mitochondrial membranes. LND was also used as an anti-spermatogenic compound because of its ability of inhibiting germ-cell respiration.

3-bromopyruvic acid (3BP) is a synthetic bromo-halogenated compound derived from pyruvic acid. Initially, 3BP was described as selectively inhibiting HK2 activity (Ko et al. 2001). Recently, however, it has been shown to exhibit stronger inhibitory activity on GAPDH, another enzyme

of the same pathway, responsible for the conversion of G3P into 1,3BPG (Ganapathy-Kanniappan 2009).

DCA and MET were chosen because of their ability to connect energy and biomass production pathways.

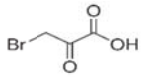
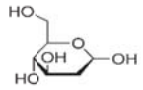
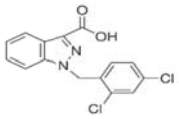
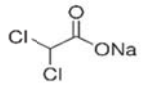
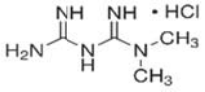
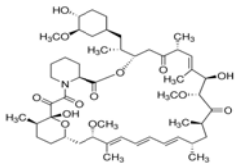
Dichloroacetic acid (DCA) is a simple compound, easy to find in drinking water in low concentration, as it is a by-product from the chlorination disinfection process for water (Villanueva et al. 2003). The main target of DCA is pyruvate dehydrogenase kinase (PDK) (Stacpoole et al. 1998). PDK inhibits the activity of pyruvate dehydrogenase complex (PDH) which regulates the entrance of pyruvate to the mitochondrion and its transformation to acetyl-CoA to start the citric acid cycle. Thus, when DCA inhibits PDK it is redirecting pyruvate to mitochondrion and restoring aerobic respiration (Michelakis et al. 2008).

Metformin (MET) is a biguanide compound that was initially developed as a possible treatment against malaria, albeit its ability to decrease blood sugar levels and the discovery of more powerful candidates to fight Plasmodium resulted in its employment for type II diabetes control. MET is thought to block the mitochondrial respiratory-chain complex I, therefore inducing unbalanced energy production (El-Mir, Fontaine et al. 2000; Owen et al. 2000), and to stimulate the energetic stress biosensor AMP-activated protein kinase (AMPK) (Fryer et al. 2002).

Sirolimus (SIR), also known as rapamycin, is a macrolide antibiotic, naturally synthetized by *Streptomyces hygroscopicus* (Sehgal et al. 1975). First used as an antifungal compound, its antitumor properties have also been demonstrated (Xu et al. 2005; Cirstea et al. 2010). In mammals, SIR specifically inhibits the TOR complex 1 (mTORC1), thereby triggering autophagic processes (Jung 2010) and down regulating the expression of several metabolic intermediates (Mita et al. 2003). However, an interference with mTORC2 can be also observed in chronic administrations of this compound (Sarbassov et al. 2006; Delgoffe et al. 2010). TOR orthologs have also been described in trypanosomatids, but a distinctive feature is that these parasites have four known TOR complexes instead the two shared in the majority of eukaryotes (Barquilla et al. 2008; Madeira da Silva and Beverley 2010; Saldivia et al. 2013). Apart from its great potential for drug repurposing in therapies against these pathogens, SIR may also be an

interesting candidate for improving vaccine efficacy due to its immunomodulatory ability, as is described in the third chapter of this work.

Table 5: Selected drugs and their energetic and biomass production-related targets

FORMULA	DRUG	LINEAR FORMULA	Energy and biomass production-related target
	3-Bromopyruvic acid	$BrCH_2COCOOH$	-GAPDH (Dell'Antone 2009) -Hexokinase 2 (Ko et al. 2001)
	2-Deoxy-D-glucose	$C_6H_{12}O_5$	Glycolysis (Nirenberg and Hogg 1958)
	Lonidamine	$C_{15}H_{10}Cl_2N_2O_2$	Hexokinase I (Paggi et al. 1988)
	Sodium dichloroacetate	$Cl_2CHCOOH$	Pyruvate dehydrogenase kinase (Michelakis et al. 2008)
	Metformin	$NH_2C(=NH)NHC(=NH)N(CH_3)_2 \cdot HCl$	-Complex I of the oxidative phosphorylation (Owen et al. 2000) -AMP-activated protein kinase (Fryer et al. 2002).
	Sirolimus	$C_{51}H_{79}NO_{13}$	mTOR (Mita et al. 2003)

4.2 New enhancing vaccine strategies: VL as a model

One of the major hurdles for developing vaccines, either to prevent or to treat VL, has been the limited understanding of the precise immune mechanisms required for controlling parasite growth, without causing disease. Because of the intrusive techniques required to analyze most tissues in VL patients, our current understanding of the host immune response during VL largely derives from studies performed in *Leishmania*-infected genetically susceptible mice (Nieto et al. 2011).

4.2.1 Immune response to *Leishmania*

Once inoculated into the skin, *Leishmania* promastigotes interact with resident dendritic cells, macrophages and γδ T lymphocytes. These cell populations recognize the pathogen through toll-like receptors (Janeway and Medzhitov 2002; Tuon et al. 2008), and secrete chemokines and cytokines that recruit neutrophils, monocytes, macrophages and natural killer cells to the site of infection (Teixeira et al. 2006). *Leishmania* is an obligated intracellular parasite with macrophages being the main host cell. However, other phagocytic cells are susceptible of being infected, such as neutrophils and dendritic cells (DCs). Neutrophils are the first cells to be recruited to the infection site, and their role in *Leishmania* pathogenesis is complex and not completely elucidated. It is known that neutrophils combat extracellular parasites by excreting nuclear DNA associated with proteins to the extracellular milieu in a so-called NETosis process (Guimarães-Costa et al. 2009). Neutrophils can also have a detrimental role in the fight against the parasite, as these cells can also get infected and act as "Trojan horses" when they are subsequently phagocytized by macrophages, and their apoptotic bodies suppress the activation of macrophages and DCs (Peters et al. 2008). In the case of monocytes, infection leads to a strong respiratory burst that activates the production of reactive oxygen species (ROS), efficiently killing the phagocytosed parasite (Novais et al. 2014). In contrast, the respiratory burst experienced by macrophages is not enough to accomplish this task, and an external source of IFN-γ is needed to potentiate its killing effect (Scott and Novais 2016). However, *Leishmanias* are well adapted to their host cell and seek to survive by developing several evasion mechanisms of the immune response, which lead to the impairment of the parasite-killing activity of the macrophage (Burchmore and Barrett 2001; Gupta et al. 2013). Monocyte-derived DC and DC migrate to lymphoid tissue, where they will initiate the adaptive immune response by activating T helper lymphocytes (Reis e Sousa 2004; Scott and Novais 2016). DCs are able to produce the pro-inflammatory cytokine IL-12, which activates and induces differentiation of different immune cell subsets such as naïve CD8+ and CD4+ T cells, and natural killer cells (NK) (Schleicher et al. 2007). NK cells develop a fundamental role during early infection, as they are an early source of IFN-γ (Laskay et al. 1993).

The majority of research has been conducted on experimental mouse models infected with the cutaneous species, *L. major*. In this model, protective responses against the parasite are dependent on the development of a type I immune response. This immune response is characterized by the generation of CD4+ T-helper (Th) 1 (Th1) in the presence of IL-12 secreted

by APCs. These T cell subsets collaborate by secreting the pro-inflammatory cytokines interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α), which are responsible for macrophage-classical activation (M1 macrophages) (Kaye and Scott 2011). These M1 macrophages are able to produce the intracellular-pathogen clearance molecule, nitric oxide, through an increased expression of inducible Nitric Oxide Synthase (iNOS2) (Bogdan et al. 1990; Kaye and Scott 2011). Conversely, Th2 lymphocytes induced by IL-4 producing APCs produce the Th2 cytokines IL-13, IL-10, IL-4 and IL-5 that promote B cell immunoglobulin production and also lead to the alternative activation of macrophages (M2 macrophages). IL-10 is known to deactivate parasite killing mechanisms of the macrophages, and to favor T cell dysfunction by suppressing antigen presentation and down-regulating IFN-γ production by T cells (Ito et al. 1999). M2 macrophages show an increased activity of arginase-1, which is associated with a leishmaniasis non-healing phenotype. Arginase-1 competes with iNOS for L-arginine —the amino acid precursor of NO— for polyamines production (reviewed by Rath et al. (Rath et al. 2014)).

In contrast to that described in cutaneous leishmaniasis, VL in mice is characterized by a compartmentalized immune response with organ-compartmentalized immunity (Engwerda and Kaye 2000; V Rodrigues et al. 2016). In the liver, parasites multiply in Kupffer cells within the first four weeks. Then, a strong cell-mediated immunity controls parasite growth. This process is associated with the development of hepatic granuloma and leads to parasite elimination and liver resistance (Murray 2001). Granulomes are formed by central Kupffer cells and surrounding T lymphocytes, which potentiate the parasite killing activity of Kupffer cells through IFN-γ secretion (Murray 2001). TNF-α also plays an important role in granuloma maturation (Kaye et al. 2004). However, parasites persist in mouse spleen, which remains chronically infected, but not killing the host (Engwerda et al. 2004). In murine spleen there is a mixed pro- and anti-inflammatory immune response, and a balance between them is needed to avoid tissue lesion (Stanley and Engwerda 2007a). Parasite persistence in spleen causes splenomegaly due to spleen remodeling, atrophy of lymphoid follicles, and hypertrophy of the red pulp (Engwerda et al. 2004). Mouse resistance is related to the *Slc11a1* gene (formerly known as NRAMP1) that controls *Leishmania* growth by the privation of required ions for parasite growth in the parasitophorous vacuole (Kaye et al. 2004).

Unlike experimental VL, human VL does not present that much evident immune compartmentalized pattern, and parasite burden increases progressively in liver, bone

marrow, and spleen during infection (Faleiro et al. 2014). In humans, the vast majority of infected people develop asymptomatic infections, which correlates with a strong CMI. However, susceptible individuals develop progressive disease with increasing parasite burdens despite a strong Th1 cytokine production (Nylén et al. 2007), suggesting there is an unresponsiveness to Th1 stimuli instead of a suppressed Th1 response. Most studies indicate there is not a clear Th2 skewing in human VL, and also point to TGF-β (Wilson et al. 1998) and IL-10 (Gautam et al. 2011) as cytokines involved in pathology and disease progression (reviewed in Kumar and Nylén (Kumar and Nylén 2012)). Although these cytokines can be produced by several immune cell types, T regulatory cells (Tregs) stand out due to their ability to control and suppress the activity of other immune components. Most Treg cells express the FoxP3 ligand and have been associated with disease progression and high production of TGF-β (Wilson et al. 1998). Paradoxically, in an inflammatory milieu, TGF-β also induces naïve CD4+ T cells differentiation towards a Th17 phenotype (Veldhoen et al. 2006), able to produce IL-17 that acts synergistically with IFN-γ to potentiate NO production in infected macrophages (Nascimento et al. 2015). Chronic VL is also associated with the presence of another Treg subset known as Tr1 cells that derive from naïve CD4 T cells that have been activated in the presence of IL-10 (Groux et al. 1997). Tr1 cells produce both IFN-γ and IL-10, and represent the main source of IL-10 during VL in spleen (Nylén et al. 2007) (Figure 11).

The hamster has been described as a faithful model for VL studies, due to its ability to mimic the human development of the disease —hepatomegaly and splenomegaly, anemia, hypergammaglobulinemia, cachexia, and immunodepression— (Requena et al. 2000; Dea-Ayuela et al. 2007). Hamster progressive VL is also accompanied by the development of hepatic granulomas, hypoplasia of the splenic white pulp, amyloid deposition in liver and spleen, and deposition of immune complexes in kidneys that provoke glomerulonephritis (Nieto et al. 2011). As in humans, a heterogeneous immune response against viscerotropic strains of Leishmania is present in the hamster models, , where pro-inflammatory cytokines —IFN-γ, IL-2, and TNF-α— have been recurrently described to be produced during VL progressive disease in hamsters in spleen concurrently with anti-inflammatory cytokines —TGF-β, IL-10 and limited induction of IL-4— (Melby, Chandrasekar, et al. 2001; Garg and Dube 2006; Loría-Cervera and Andrade-Narváez 2014). Hamster susceptibility is primarily due to the resistance hamsters show to the induction of the iNOS activity by IFN-γ (Bories et al. 1998; Melby, Chandrasekar, et al. 2001; Perez et al. 2006). This characteristic makes hamsters highly susceptible to *Leishmania* infection, which is further aggravated by the apparent lack of

lymphoproliferative responses of this animal model once infected (Nieto et al. 2011; Loría-Cervera and Andrade-Narváez 2014), as also happens in humans. Nevertheless, hamsters are not common as animals for research and thereby the lack of tools for analysis limits the range of available information and restricts the design of the experimental phase.

Little is currently known about the role of CD8+ T cells during leishmania infection, but evidence seems to indicate that these cells are involved in a protective response against both visceral and cutaneous presentations. These cells produce TNF-α, IFN-γ, and cytotoxic molecules in response to IL-12 (Henry et al. 2008; Kaech and Cui 2012) (figure 11). CD8+ T cells control parasite growth in experimental VL, and are involved in resistance to re-infection after completed healing or therapeutic vaccination (Stäger and Rafati 2012; Gautam et al. 2014; Kaushal et al. 2014). Increased CD8+ T cell levels have also been associated with asymptomatic *Leishmania* infections (Reis et al. 2010), whereas a depletion of these lymphocytes during a vaccine trial abrogated the protection achieved (Gurunathan et al. 1997), therefore providing evidence of their fundamental role in a protective response. Furthermore, it is known that *L. donovani* VL impairs clonal expansion of CD8+ T cells responses that has been suggested to be due to the low availability of *Leishmania* antigens, since the parasite abrogates antigen processing and presentation (Stäger and Rafati 2012; Matheoud et al. 2013), and the concurrently enhanced expression of the inhibitory receptors in CD8+ T cells (Gigley et al. 2012). In addition, CD8+ T cells have been suggested as contra-Treg cells during *L. donovani* VL due to their ability to limit IL-10 production (Martin et al. 2010).

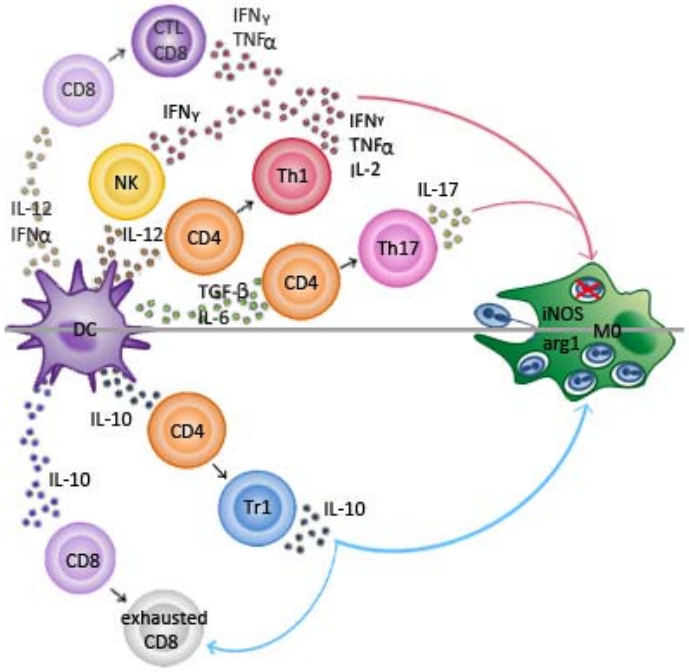


Figure 11: Main host immune response during visceral leishmaniasis in spleen. Pro-inflammatory (top) and regulatory and inducer of parasite persistence (bottom) environments are represented and divided by a gray line

4.2.2 Our vaccine approach

As described elsewhere in this work, the administration of DNA sequences represents a useful tool in vaccine development. This strategy consists in the insertion of DNA sequences that codify for an antigen of interest in a plasmid vector. Once this structure is administered to the subject, APCs will be able to express the protein of interest (Wolff et al. 1990), which will be processed for stimulating humoral and cellular immune responses (Huygen 2005). In addition, plasmid vectors also act as immune adjuvants. This characteristic is conferred by the natural presence of unmethylated CpG motifs in prokaryotic DNA. These bacterial sequences trigger Toll-like receptor 9 activation in APCs (Hemmi et al. 2000), therefore boosting Th1 responses by promoting the non-specific production of IFN-γ and CD4+ and CD8+ responses (Gurunathan, Klinman, et al. 2000; Huygen 2005). The suitability of DNA vaccines also relies on the low cost production and easy storage, as these vaccines are stable and no cold preservation is needed (Gurunathan, Klinman, et al. 2000). Also, these vaccines are very safe due to the lack of pathogenic organisms in the inoculum, which ensures its non-virulence (Gurunathan, Klinman, et al. 2000).

Several DNA vaccination approaches against *Leishmania* have been tested to date, reaching different levels of protection that could be enhanced with the use of a proper adjuvant. Our research group has previously studied the effect of a naked DNA vaccine based on a pVAX plasmid vector carrying the following *Leishmania* genes: Tryparedoxin Peroxidase (*TRYP*), *Leishmania* homolog of receptors for Activated C kinase (*LACK*), Potentially Aggravating Protein of *L. infantum* (*PAPLE22*) and Kinetoplastid Membrane Protein-11 (*KMPII*) (Todolí et al. 2012) (figure 12).

TRYP, initially known as TSA protein, due to its resemblance to eukaryotic thiol-specific-antioxidant protein (Webb et al. 1998), and currently also called TXNPx, is an enzyme with an important role in the protection of the parasite from oxidative damage by catabolizing hydrogen peroxide (Levick et al. 1998). It is highly conserved across *Leishmania* spp., and its activity is linked to parasite survival, virulence, and drug response (Iyer et al. 2008; Henard et al. 2014).

LACK is a highly conserved antigen among *Leishmania* parasites, and is widely known for being recognized by T cells and promoting non-protective Th2 in the susceptible murine model of *L. major* CL (Schilling and Glaichenhaus 2001). In *Leishmania* parasites responsible for causing VL,

this protein is also highly conserved and presents low variability and high immunogenicity (Sinha et al. 2013). In *L. infantum* LACK is located in the cytosol close to the kinetoplast, and possibly interacts with sequences from proteins involved in DNA replication and RNA synthesis (Gonzalez-Aseguinolaza et al. 1999). In addition, LACK plays an essential role for parasite viability and vertebrate parasitization (Kelly et al. 2003).

PapLE 22 is an endogenous nuclear protein detected in several *Leishmania* species. This antigen is known to contribute to VL pathogenesis and immunosuppression by stimulating IL-10 production (Suffia et al. 2000).

KMPII, also known as KMP-11, is a surface protein located throughout the parasite surface with more presence around the flagellum and flagellar pocket. Its expression depends on the stage and species of the parasite, being highly expressed in *L. infantum* promastigotes (Berberich et al. 1998; Lynn et al. 2013). KMPII is suggested to play a role in membrane stabilization (Jardim et al. 1995), and its downregulation has also been associated with antimonial resistance (El Fadili et al. 2009). This protein is known to be a potent stimulator of T (Tolson et al. 1994) and B cells (Jensen et al. 1998), also playing an important role in infectivity and as a virulence factor (de Mendonça et al. 2015).

These DNA sequences code for proven immunogenic antigens shown to induce protection in rodent models of *Leishmania* infection (Mutiso et al. 2013; Joshi et al. 2014; Kumar and Engwerda 2014). It is expected that using a DNA multicomponent vaccine coding for these *Leishmania* antigens will enhance the spectrum of the immune response. However the vaccine induced a poor immunogenic response when administered alone, but was protective when used in a prime-boost strategy (Todolí et al. 2012).

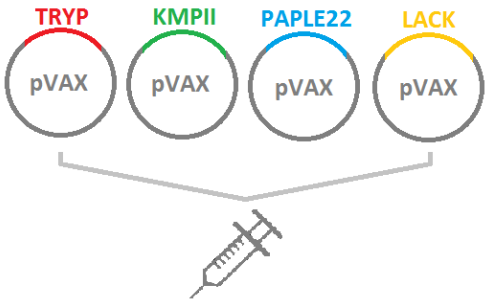


Figure 12: Vaccine designed in our laboratory: *L. infantum TRYP*, *KMPII*, *LACK* and *PAPLE22* gene sequences inserted in pVAX plasmids.

4.2.3 Immune modulation as a tool for combating and preventing VL

The host immune response plays a pivotal role in the outcome of the infection and in the efficacy of treatments against *Leishmania*. Immunomodulators are substances that enhance or downregulate the immune system activity. The use of these compounds may redirect the immune response to control pathogen infections, as is the case of *Leishmania* (Taslimi et al. 2016). Immunomodulators can be administered alone as immunochemotherapy —as is the case of domperidone (Gómez-Ochoa et al. 2009) or imiquimod (Buates and Matlashewski 1999)— or in combination with other chemotherapies (Reviewed in Dalton and Kaye (Dalton and Kaye 2010)), vaccines, or both (Reviewed by Roatt et al. (Roatt et al. 2014)).

In the case of vaccines it is common to use interleukins and other immunomodulatory compounds as adjuvants. Many adjuvants have been used in *Leishmania*-vaccine trials, achieving variable success. Th1 immune response has been induced by Bacille de Calmette-Guérin, aluminum salts (Misra et al. 2001), the IL-12 cytokine (Afonso et al. 1994), and saponins (Borja-Cabrera et al. 2008), showing to increase the immunogenic power of the vaccine which they were assayed. The main problems related to the choice of a suitable adjuvant for vaccination are physical-chemical incompatibilities with the vaccine content or with other adjuvant, and undesirable reactions associated with their administration (Reviewed by other authors (Raman et al. 2012; Mutiso et al. 2013)).

4.2.4 SIR as a combined immunomodulatory candidate for VL prophylactics

SIR is an immunosuppressant licensed and marketed drug that is prescribed to transplant recipients to prevent allograph rejections. Its immunomodulatory activity is due to specific inhibition of mTOR that produces from immunostimulatory to immunosuppressive effects, depending on the cell subset and on the time of administration during the development of a specific or acquired immune response. Recent studies have opened a whole new field of research by showing that the administration of SIR during the expansion and concentration phase of a primary immune response resulted in an increased magnitude and quality of CD8+ T memory cells (Araki et al. 2009). During acute infection —or vaccination—, inflammatory signals and antigenic stimulation induce antigen-specific CD8+ T cell clonal proliferation. This initial stage is termed as the expansion phase of the immune response and culminates within the following 10 days with the creation of a CD8+ pool. The vast majority of these cells become

short-lived effector CD8+ T cells, and only a small percentage of these original naïve CD8+ T cells become memory precursor effector cells. During the next 20 days, there is a selection of these CD8+ expanded cells, where most of them enter into an apoptotic state in the so-called contraction phase of the immune response. However, memory precursor effector cells survive, and with time, developing to become self-renewing long-lived memory T cells (Araki et al. 2010) (Figure 13). In an elegant study, Araki et al. showed that the modulation of the mTOR route by administering sirolimus in different phases of development of CD8+ T cells response induces an increase in quantity, quality, or both, of CD8+ T memory effector cells (Araki et al. 2009).

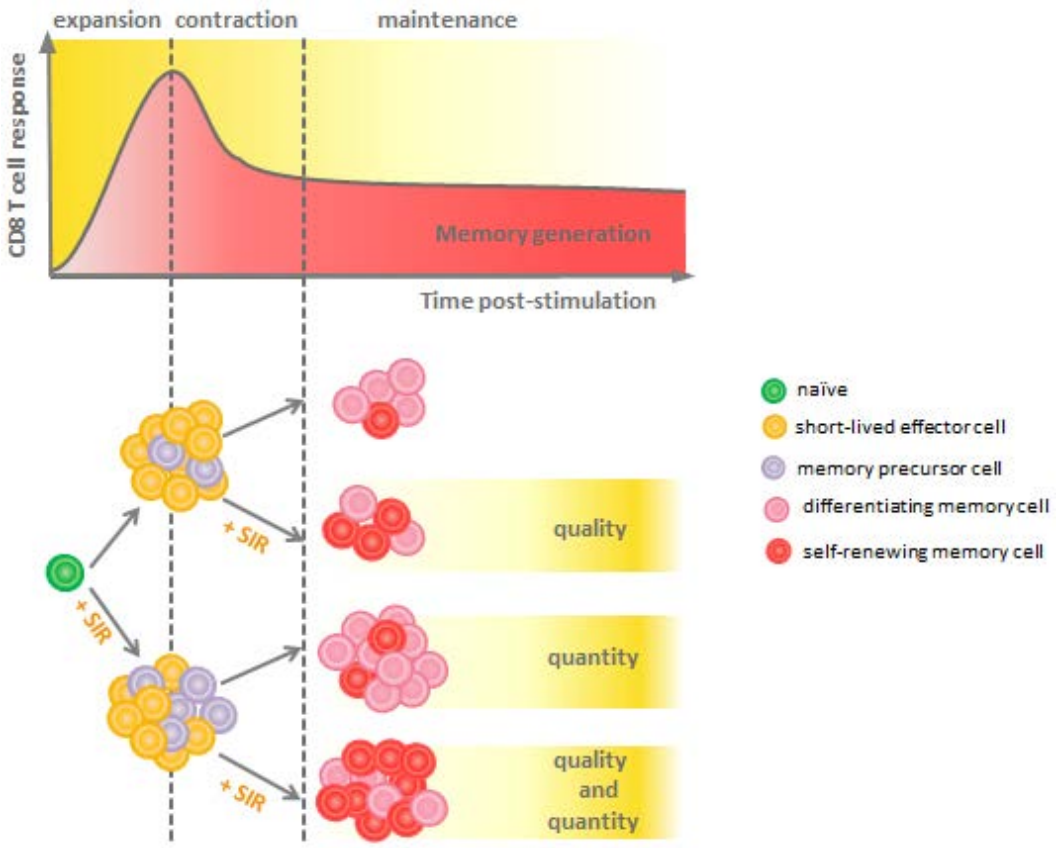


Figure 13: Memory CD8+ T cell differentiation and the influence of sirolimus administration during the expansion and contraction phase of the immune response. Modulation of the mTOR route by administering sirolimus in different phases of development of CD8+ T cells response induces an increment in quantity, quality, or both, of CD8+ T memory effector cells. Modified from Araki et al. (Araki et al. 2010).

Administration of SIR has shown promising results on different vaccine trials against viruses (Turner, Shaffer, et al. 2011; Keating et al. 2013), cancer models (Li et al. 2012; Diken et al.

2013) and even intracellular mycobacteria (Jagannath and Bakhru 2012). SIR also enhance the survival of CD8+ T antigen-specific cells (Araki et al. 2009), and of DCs when administered during TLR activation by avoiding the switch from energetic cellular metabolism to aerobic glycolysis (Amiel et al. 2012). Thus, these long-lasting DCs can stimulate T cells longer than the glycolysis-committed ones, therefore inducing greater T cell responses (Amiel et al. 2012). Furthermore, SIR is well known for positively regulating autophagic processes (Jung 2010) promoting T cell stimulation by increasing antigen presentation by MHCII molecules on APCs (Jagannath et al. 2009). In addition, SIR has been linked to cytokine production and CD4+ T effector cell differentiation, since SIR-treated macrophages and DCs increase production of the pro-inflammatory cytokine IL-12, and reduce the release of IL-10 under TLR stimulation (Ohtani et al. 2008; Weichhart et al. 2008). Taken together, this information suggests that the effects of SIR over CD4+ T cells and CD8+ T cell memory store quality in a vaccine trial may lead to the promotion of a Th1 response and a rapid re-call of CD8+ T cells mediating protective immunity against the disease. In the current work, the immune modulatory compound SIR was used for the first time to boost the immune response induced by the previously described DNA vaccine against *Leishmania*.

HYPOTHESIS

& AIMS

HYPOTHESIS

“Protozoan parasites have high metabolic demands owing to their rapid multiplication in the mammalian host cell. Trypanosomatids and *Plasmodium spp* metabolism largely depend on glycolysis to support their high energetic requirements. Therefore, metabolic pathways involved in energy production in protozoan parasites and their modulation could be possible therapeutic targets. Drugs that modulate these pathways may induce a reduction on parasite growth and viability slowing down the infection process and even lead to the complete clearance of the parasite. Furthermore, the modulation of these pathways in immune system cells may contribute to boosting the efficacy of a vaccine by improving the immune response to the infection**”**.

AIMS

The overall aim of this thesis has been to evaluate the efficacy of compounds known to directly or indirectly modulate energy production metabolic pathways in protozoan parasites, and their possible use as vaccine adjuvants. For that purpose, we aim to validate selected inhibitors of glycolysis and related pathways for treating and preventing leishmaniases, malaria, and American and African trypanosomiasis.

We established 3 different objectives to tackle this topic:

To test i*n vitro* the efficacy of drugs acting on bioenergetic pathways in the management of visceral leishmaniasis, American and African trypanosomiasis, and malaria.

To study the efficacy of bioenergetic modulators selected from the previous chapter in *in vivo* models of visceral leishmaniasis, American and African trypanosomiasis, and malaria.

To determine if the administration of sirolimus during the immunization with a DNA vaccine may protect against experimental challenge with *L. infantum.*

In vitro assay of bioenergetic modulators

Summary of the experimental approach:

This chapter is destined to tackle the first objective of this work:

"To test *in vitro* the efficacy of drugs acting on bioenergetic pathways in the management of visceral leishmaniasis, American and African trypanosomiasis, and malaria"

To initially assess the aptitude of the known compounds 2-deoxy-D-Glucose, 3-bromopyruvic acid, lonidamine, sirolimus, dichloroacetic acid and metformin to manage malaria, visceral leishmaniasis, and African and American trypanosomiasis, we first studied their associated *in vitro* effect. For that purpose, we tested the cytotoxicity of these compounds to host cells of each *in vitro* culture model, determining the range of possible therapeutic concentrations. Once established, these compounds were assayed on *in vitro* cultures of *L. infantum* promastigotes, intra-macrophagic *L. infantum* amastigotes, intra-cellular *T. cruzi* amastigotes, bloodstream *T. brucei* trypomastigotes, and *P. falciparum*-infected red blood cells. Parasites were co-cultured for 48–72 h with the studied compounds and their efficacy was recorded as the ability of each one to reduce available parasite forms. IC_{50} values were calculated and compared between models and with first choice drugs.

MATERIALS AND METHODS

Parasites and Mammalian Cell Cultures

L. infantum JPC strain (MCAN/ES/1998/LLM-724) was kindly provided by Dr. Javier Moreno and Dr. Eugenia Carrillo, ISCIII Madrid, Spain. *L. infantum* promastigotes were cultured at 26 ºC in R15 medium [RPMI 1640 medium (Gibco) supplemented with 15 % heat-inactivated fetal bovine serum (FBS) (Gibco), 2 % HEPES 1M (Gibco), 1 % 10000 U/mL penicillin, and 10 000 µg/mL streptomycin (Gibco)]. Weekly passages were performed. Metacyclic promastigotes for *in vitro* infections were obtained from a 6-day-old stationary culture. U937 human macrophages derived from a Caucasian histiocytic lymphoma were obtained from the European Collection of Cell Cultures (ECACC). Cells were cultured in RU937 media (RPMI-1640 medium supplemented with 10 % FBS and 1 % Penicillin/Streptomycin), and kept in a humid atmosphere at 37 °C and in 5 % CO_2. Before carrying out each experiment, 1×10^6 U937 cells were seeded in a 25 mm^2 culture flask and differentiated in a monolayer by adding Phorbol 12-myristate 13-acetate (PMA) at a final concentration of 50 nM for 48 h, and then washed twice with phosphate buffered saline (PBS) ×1, and fresh media added (Maia et al. 2007).

P. falciparum 3D7 (obtained from MR4-ATCC) chloroquine-sensitive parasites were cultured with human red blood cells (RBCs) obtained from the Blood and Tissue Bank (Catalonia, Spain), after approval from Hospital Clínic of Barcelona's Clinical Research Ethics Committee. Parasites were co-cultured with B^+ RBCs (3 % hematocrit) in RPMI medium with 10 % human AB^+ type serum, and kept at 37 °C in an atmosphere of 93 % N_2, 2 % O_2, and 5 % CO_2. In order to select ring stage parasites, a sorbitol synchronization protocol was followed, keeping infected erythrocytes at 37 ºC for 10 min with 10 times its volume of sorbitol 5 % (W Trager and JB Jensen 1976).

The *T. brucei brucei* 427 strain was maintained in HMI-9 culture medium [Iscove's modification of DMEM (IMDM; Cell Gro) supplemented with 10 % FBS, 10 % Serum plus (SAFC), 0.05 mM Bathocuproine sulfonate, 1.5 mM L-cysteine, 1 mM hypoxanthine, 0.2 mM β-mercaptoethanol, 0.16 mM thymidine 1mM pyruvate)], and passages were performed twice weekly.

CL strain *T. cruzi* overexpressing a tdTomato red fluorescent protein was used (Canavaci et al. 2010). Parasites were maintained in Vero cell cultures through weekly passage. Fibroblast-like

kidney cells from African green monkey (Vero cells, ECACC) were purchased from Sigma Aldrich (Ref 84113001). Vero cells were maintained in culture media [RPMI 1640 supplemented with 10 % fetal bovine serum, gentamycin (25 µg/mL), Penicillin/Streptomycin (0.1 mg/mL), L-glutamine (2 mM), sodium pyruvate 1 mM), and 2-mercaptoethanol (50 µM)] at 37 °C and in 5 % CO_2. Passages were performed twice weekly. After 4–5 days of culture, trypomastigotes released from Vero cells were collected to carry out the drug screening assay.

Drugs

3BP (CAS N1113-59-3), DCA (CAS N 79-43-6), 2DG (CAS N 154-17-6), LND (CAS N 50264-69-2), MET (CAS N 1115-70-4), and SIR (CAS N 53123-88-9) were purchased from Sigma-Aldrich and freshly prepared before use. The following reference drugs were selected as positive controls: chloroquine for *P. falciparum*, benznidazole for *T. cruzi*, Glucantime (meglumine antimoniate, Sanofi-Avensis) for *L. infantum* intracellular amastigotes, amphotericin B (AmB) for *L. infantum* promastigotes, and suramin for *T. brucei in vitro* experiments.

Cytotoxicity to mammalian cells

In order to test toxicity on human macrophages, 5×10^4 U937 differentiated cells per well were seeded in 96-well microplates, and cultured with increasing concentrations of 3BP, DCA, 2DG, MET, LND, and SIR diluted in RU937 culture media. Each concentration was assayed in triplicate. The cells were kept at 37 °C in a 5 % CO_2 atmosphere. Cell viability was determined 48 h later by the MTT assay. Briefly, Thiazolyl Blue Tetrazolium Bromide (MTT) was added to each well at a final concentration of 0.5 mg/mL. After 4 h of incubation in the dark, 100 µL of HCl 0.1 N in isopropanol + 10 % Triton X-100 was added to each well to solubilize the resulting formazan crystals, and then read at a wavelength of 570 nm (reference 690 nm). The Alamar Blue (Invitrogen) assay was performed in the case of 3BP, in order to avoid interferences (Ganapathy-Kanniappan et al. 2010). Alamar Blue was added in an amount equal to 10 % of the culture volume, and plates were kept in the incubator for 24 h. Absorbance was read at 570 nm and 600 nm, and results were calculated following the manufacturer's recommendations.

For cytotoxicity assays on Vero cells, 1.7×10^4 cells were seeded in black, clear-bottom 96-well plates and incubated overnight at 37 °C and in 5 % CO_2. Serial dilutions of the drugs were

added and incubated at 37 °C and in 5 % CO_2 for 72 h. Alamar Blue (AbD Serotec) was then added and the measurements performed 6 h later as described above.

In order to evaluate the compatibility of the selected drugs and erythrocytes, hemolysis was studied *in vitro* by direct spectrophotometry. RBCs (3 % hematocrit) were cultured in round-bottom 96-well plates and incubated for 48 h with serial dilutions of the studied compounds in a humid atmosphere with concentrations of 5 % CO_2, 2 % O_2, and 93 % N_2. Each sample was assayed in duplicate, and a positive control for hemolysis was included by adding autoclaved distilled water to a well containing RBCs. The Harboe method was used to assess hemolysis (Harboe 1959).

Effect of bioenergetic modulators on *L. infantum*

The killing effect of these drugs was assessed against the two stages of the biological cycle of *Leishmania*: motile promastigotes in the sand-fly vector and intracellular amastigotes in the host.

- Anti-*L. infantum* promastigotes drug test

Promastigotes from a 4-day culture were seeded at 1×10^5 parasites per well in a 96-well microplate with increasing concentrations of drugs for 48 h at 26 °C. AmB at 0.25 µM was used as positive control. In order to determine parasite viability, the phosphatase activity assay was performed. Briefly, parasites were completely lysed using a solution made up of 1 % Triton X-100 and 2.9 % sodium citrate in distilled water. Then, 5 mM of p-nitrophenyl phosphate was added to each well. In viable cells, p-nitrophenyl phosphate was hydrolyzed by intracellular acid phosphatases to p-nitrophenol. After 2 h incubation at 37 °C, the reaction was stopped by adding 60 µL of NaOH 1 N to each well. Data were collected by reading the optic density at a wavelength of 405 nm (Yang et al. 1996), and standardized based on the optical density obtained for the untreated wells. Results were expressed as the survival rate of promastigotes under treatment conditions.

-Drug test on *L. infantum* intra-macrophage amastigotes

Cell line U397 was cultured in 25 mm^2 flasks and differentiated as explained above. Metacyclic promastigotes (6 days' culture) were added to the pre-washed differentiated cell culture at a parasite:cell ratio of 10:1, and co-cultured for 24 h. They were then washed with PBS 1× to discard non-internalized promastigotes. Infected macrophages were re-suspended in RU937 and incubated in 8 well chamber slides at a density of 5×10^4 with increasing, but non-toxic to human macrophages, concentrations of 3BP, DCA, 2DG, SIR, LND, and MET for 48 h. Glucantime at a concentration of 100 µg/mL was used as positive control. Each concentration was tested in duplicate. Preparations were fixed with methanol and stained with Giemsa 11 %. The numbers of infected macrophages and the intracellular parasites were recorded by direct microscopic count of 200 cells per sample. Values of infected macrophages and parasite burden were expressed as a rate based on the untreated samples. The concentration of drug needed to inhibit 50 % of control growth (half maximal inhibitory concentration 50, IC_{50}) was calculated by using GraphPad Prism Software v5.

***P. falciparum* growth inhibition assay**

RBCs were cultured in RPMI and spiked with *P. falciparum* ring stage infected erythrocytes to a final hematocrit of 3 % and 0.8 % infected blood cells. Cultures were then transferred to 96 micro-well plates and exposed to increasing concentrations of 3BP, DCA, 2DG, MET, LND, and SIR prepared in culture media. Chloroquine (CQ) (80 nM) was used as reference drug to achieve parasite growth inhibition values above 90 % (> IC_{90}). Each concentration was assayed in triplicate and kept at 37 °C in a 5 % CO_2, 93 % N_2, and 2 % O_2 atmosphere. After 48 h, parasitemia was determined by fluorescence-assisted cell sorting [FACS] (BD LSRFortessa cell analyzer), after staining infected RBCs with SYTO 11 (Molecular Probes). Non-infected RBCs and infected untreated RBCs were used as controls. The results were expressed as the inhibition rate of parasite growth in relation to the infected untreated RBCs. IC_{50} was calculated by non-linear regression by using GraphPad Prism Software v5.

Screening of drugs against *T. brucei*

Cultured parasites were collected and spun for 10 min at 900 *g* to eliminate supernatant and were then cultivated at 5×10^5 parasites per well in a 96-well white, sterile plate in warm

medium with serial dilutions of the selected compounds. Suramin (SUR) at 100 µM was used as positive control. Each concentration was tested in triplicate. After 24 h, Alamar Blue (Sigma) was added as previously described, and plates were incubated at 37 °C for 4 h before fluorescence was read at excitation (Ex) 530 nm and 590 nm emission (Em) wavelength. Non-linear regression analysis (GraphPad Prism Software v5) was used to determine IC_{50} values of tested compounds.

Drug test on intracellular amastigotes of *T. cruzi*

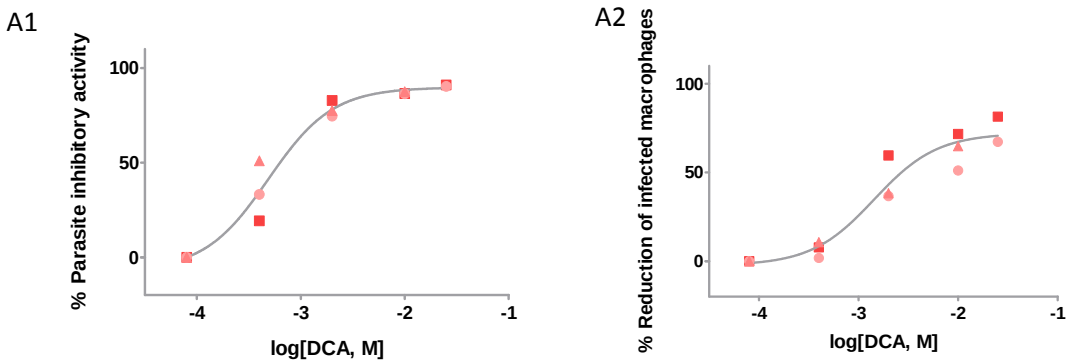
Gamma-irradiated (2,200 rad) Vero cells were plated in 96-well, black, clear-bottom plates at 3.6×10^4 cells per well and left overnight at 37 °C, in 5 % CO_2 atmosphere. Vero cells were then infected with 3.6×10^5 trypomastigotes of the CL tdTomato strain of *T. cruzi* per well for 5 h at 37 °C and 5 % of CO_2. After infection, the plates were washed once with Hanks' solution to eliminate extracellular parasites and cultured again in the presence of serial dilutions of the selected compounds. Benznidazole (BZD) was used as a positive control drug at a concentration of 2 µM. Each concentration was assayed in quadruplicate. After 72 h, fluorescence was measured (Ex = 544 nm; Em = 612 nm) and IC_{50} values were determined by non-linear regression analysis (GraphPad Prism Software v5).

RESULTS

Leishmanicidal activity of bioenergetic modulators on *L. infantum* promastigotes and intramacrophagic amastigotes.

The activity of DCA, 3BP, 2DG, LND, MET, and SIR on *Leishmania* promastigotes was assessed after culturing parasites with the drugs for 48h. Only 3BP and 2DG showed a slight parasite-killing effect at doses of 37 µM and 0.2 mM, respectively, and did not reach the values obtained by the control drug AmB (25 µM) (36 % survival rate) (data not shown). SIR, DCA, MET, and LND did not detectably impair *L infantum* promastigote survival.

When these compounds were tested in an infection assay of *L. infantum* intra-macrophage amastigotes, an inhibitory, dose-dependent effect was observed both in infected and treated cells with 3BP and DCA. The percentages of infected macrophages after 48 h of treatment with the highest, but non-cytotoxic doses of these compounds, were reduced by 74.4 % for 25 mM of DCA and 48.4 % for 75 µM of 3BP. Concurrently, the number of intracellular amastigotes decreased by 90.7 % for DCA and 78.3 % for 3BP, values 31.1 % and 18.6 % higher than those obtained with Glucantime at 100 µg/mL, respectively (Figure 14). Their parasite-killing activity is translated to IC_{50} values of 631.5 µM for DCA and 17.19 µM for 3BP. The other tested drugs only showed a slight effect, and their anti-parasitic activity was lower than that produced by Glucantime (data not shown). SIR did not show any parasiticidal effect, although a modest increase in the parasite burden and the number of infected macrophages were observed at a concentration of 10 µM (data not shown).



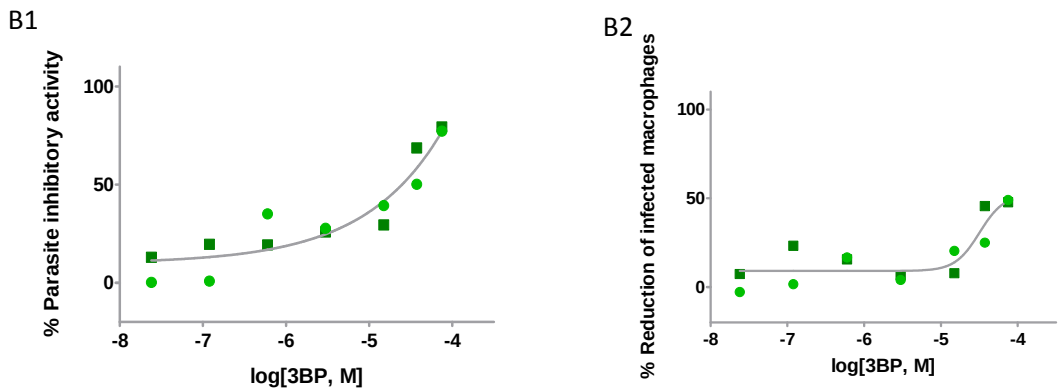
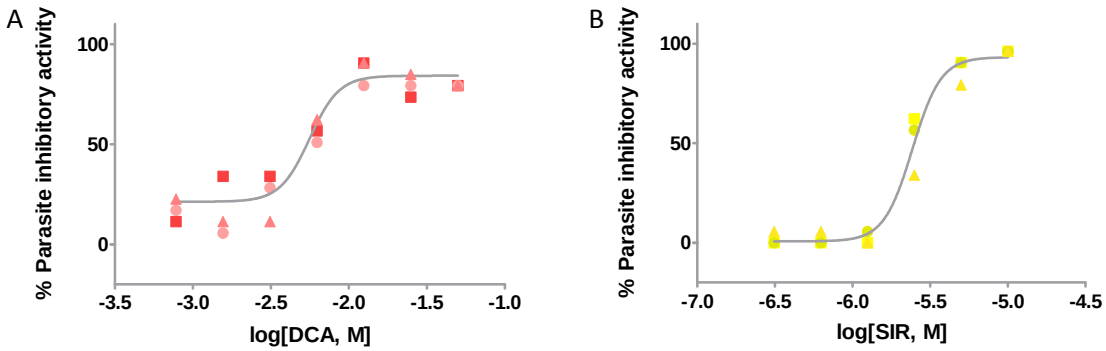


Figure 14: Efficacy of DCA and 3BP on *L.infantum*-infected macrophages. Dose response curves showing inhibition rates of *L. infantum* intracellular amastigotes growth (1) and *L. infantum*-infected macrophages (2) based on the values for the untreated controls after 48 h of treatment with (A) DCA and (B) 3BP.

Antiplasmodial activity of bioenergetic modulators

P. falciparum intra-erythrocytic stages were dose-dependent sensitive to DCA, 2DG, SIR, and MET and reached growth inhibition values close to those obtained by CQ (90 %). IC_{50} values indicate *P. falciparum* is highly susceptible to SIR (IC_{50} = 2.50 µM). DCA (IC_{50} = 5.39 mM), 2DG (IC_{50} = 4.19 mM), and MET (IC_{50} = 1.32 mM) needed higher concentrations to inhibit parasite growth. A slight effect on parasite growth inhibition was observed at high concentrations for 3BP. LND showed a peak of anti-malarial activity at 300 µM (IC_{50} = 209.13 µM) (Figure 15).



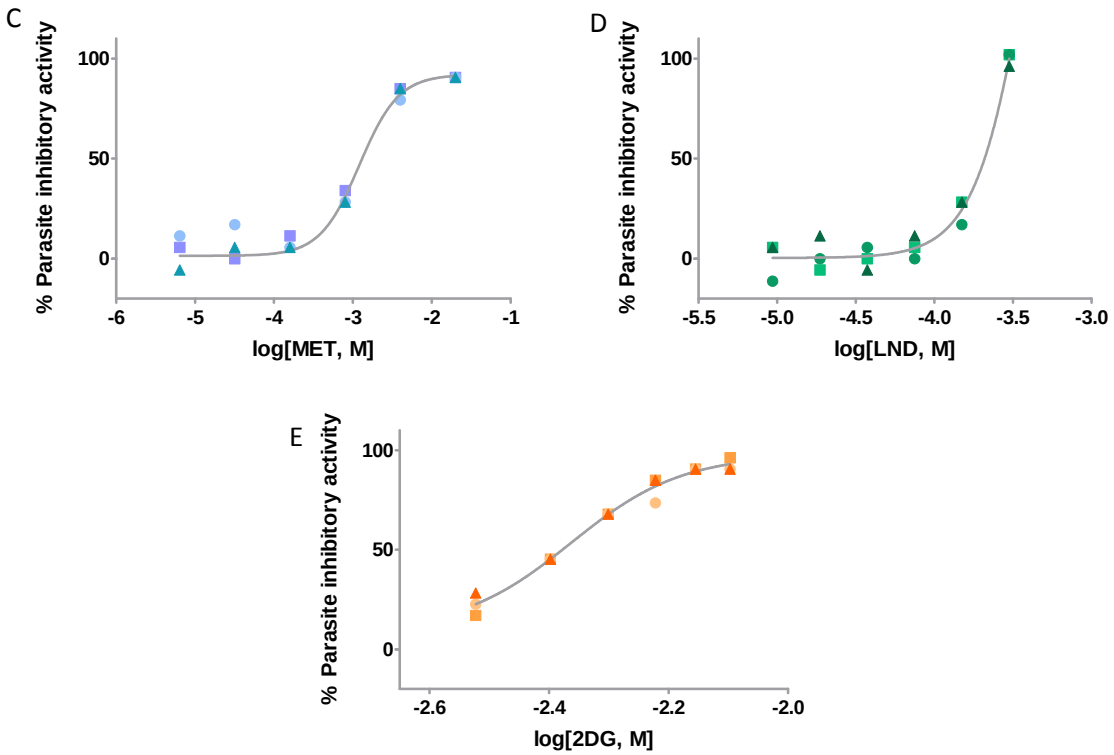


Figure 15: Growth inhibition of *P. falciparum*. Inhibitory activity of (A) dichloroacetic acid, (B) sirolimus, (C) metformin, (D) lonidamine, and (E) 2-deoxy-D-glucose on *P. falciparum in vitro* assays. Data was obtained by flow cytometry and the inhibition rate of parasite growth was then calculated with respect to infected but untreated RBCs.

Anti-*T. brucei* activity of bioenergetic modulators

DCA and SIR achieved complete clearance of cultured *T. brucei brucei* parasites at 12.5 mM (IC_{50} = 1.24 mM) and 6.85 µM (IC_{50} = 2.14 µM) concentrations, respectively. Similar values were also obtained for LND (IC_{50} = 26.76 µM) and 3BP (IC_{50} = 76.57 µM), showing 97.7 % and 93.2 % parasite-killing activity, respectively. MET only produced 57 % parasite elimination at 20 mM (IC_{50} = 17.30 mM). No parasite-killing activity was detected for 2DG. SUR 100 µM was used as a control drug, inducing nearly complete parasite clearance (96 %) (Figure 16).

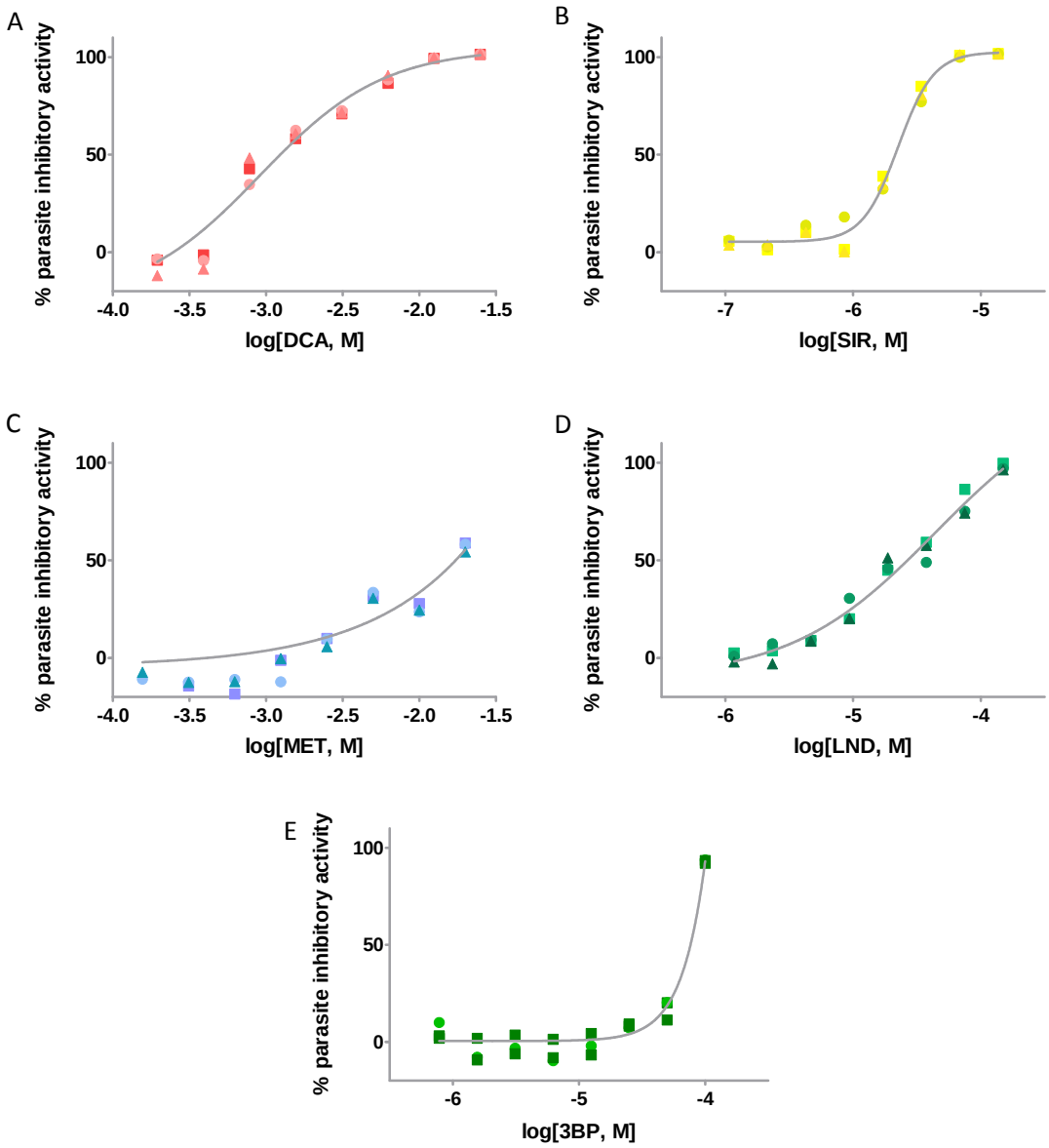


Figure 16: Dose-response curves of anti-trypanosomal activity on *T. brucei* cultured parasites. Fluorescence values, obtained after culturing parasites with (A) dichloroacetic acid, (B) sirolimus, (C) metformin, (D) lonidamine, and (E) 3-bromopyruvic acid, indicate their *T. brucei*-killing activity. Suramin was used as a positive control.

Effect of bioenergetic modulators on *T. cruzi* amastigotes

3BP, LND and 2DG produced the highest reduction in *T. cruzi* amastigote levels, reaching inhibition values of 77.8 % (IC_{50} = 27.63 µM), 99.4 % (IC_{50} = 78.37 µM) and 89.3 % (IC_{50} = 7.27 mM), respectively. DCA and MET also decreased parasite survival but did not reach the level of the positive control. DCA action required high concentrations (up to 100 mM) in order to reach the highest value of 74.1 % (IC_{50} = 27.07 mM), while MET only produced mild inhibition with a

maximum of 51.7 % at 20 mM (IC_{50} = 18.48 mM). A sustained mild reduction in fluorescence was detected at higher concentrations, which may suggest a trypanosomastatic effect (Figure 17). In contrast, SIR did not show consistent anti-*T. cruzi* activity (data not shown).

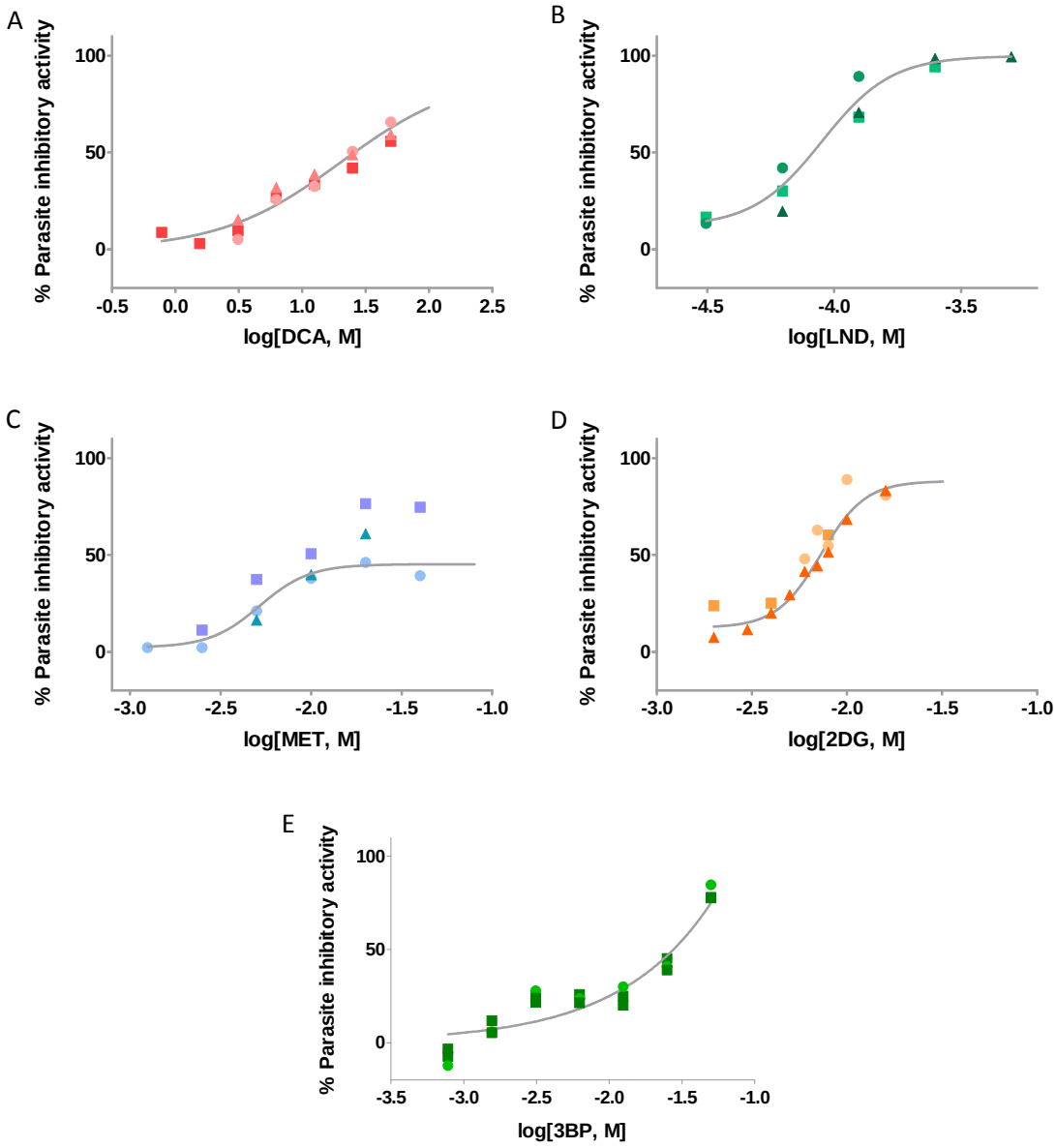


Figure 17: Parasite-killing activity on *T. cruzi* intracellular amastigotes. The reduction in emitted fluorescence represents the parasite-killing power of (A) dichloroacetic acid, (B) lonidamine, (C) metformin, (D) 2-deoxy-D-glucose, and (E) 3-bromopyruvic acid.

Table 6: Summary of IC_{50} obtained from selected drugs on *in vitro* models of studied parasites.

Compound	***P. falciparum*** intra-erythrocytic stage	***T. cruzi*** intracellular amastigotes	***T. brucei*** bloodstream stage	***L. infantum*** intracellular amastigotes	***L. infantum*** infected macrophages	***L. infantum*** promastigotes
3BP	-	27.63 µM	76.57 µM	17.19 µM	>75 µM	-
DCA	5.39 mM	27.07 mM	1.24 mM	631.5 µM	2.69 mM	-
2DG	4.19 mM	7.27 mM	-	-	-	-
LND	209.13 µM	78.37 µM	26.76 µM	-	-	-
MET	1.32 mM	18.48 mM	17.30 mM	-	-	-
SIR	2.50 µM	-	2.14 µM	-	-	-

Acronyms: 3BP (3-bromopyruvic acid), DCA (dichloroacetic acid), 2DG (2-deoxy-D-glucose), LND (lonidamine), MET (metformin), and SIR (sirolimus).

In vivo assay of bioenergetic modulators

Summary of the experimental approach:

This chapter is destined to tackle the second objective of this work:

"To study the efficacy of bioenergetic modulators selected from the previous chapter in in vivo models of visceral leishmaniasis, American and African trypanosomiasis, and malaria"

For that purpose, different approaches in four different disease models were performed.

- *Test of DCA and 2DG on the* in vivo *model of malaria.*

DCA and 2DG were selected due to their ability to kill intra-erythrocytic stages of *P. falciparum.* These compounds were assayed in the malaria murine model, where BALB/c mice were infected with a lethal strain of *P. yoelii*. Different doses of these compounds were tested in a "4 and 7-day suppressive test" to determine the parasite clearance and therapeutic efficacy. Parasitemia was examined in Giemsa stained thin blood smears.

- *Test of DCA, 3BP and MET on the* in vivo *model of American and African Trypanosomiasis.*

DCA, 3BP and MET provided good parasite killing results in *in vitro* experiments and so were selected to be assayed in the *in vivo* CD and HAT model in mice. For this, mice were infected with transgenic parasites expressing luciferase and the evolution of the disease was assessed by comparing the emitted luminescence before and after the treatment for each mouse.

- *Test of DCA and 3BP on an* in vivo *model of VL.*

In order to determine the ability of DCA and 3BP to constrain the development of VL, these bioenergetics modulators were assayed in the hamster *in vivo* model of VL. These two compounds were selected due to their ability to kill intracellular amastigotes as described in the first chapter. Hamsters were infected with *L. infantum* and, when animals reached enough parasite burdens in target organs, they were treated with doses of these compounds previously described for cancer studies. Parasite burden, histological analysis of target organs, and the study of immune markers, were determined to evaluate the evolution of the disease.

MATERIALS AND METHODS

- **Test of DCA and 2DG on the in vivo model of murine malaria**

Animals

Female BALB/c mice, 7 weeks-old were used in this experiment. Animals were housed under standard conditions in the animal housing facilities service for research purposes in the Hospital Clinic de Barcelona (Barcelona, Spain), with free access to water and food. Mice were caged in groups of 5 animals corresponding to the different groups of treatment (4 groups per assay). All procedures were performed in agreement with Article 32 of Executive Order number 214/1997 of 30th of July, on the protection of animals for research and other scientific purposes.

Parasites and infection

P. yoelii lethal strain XL was used for these experiments. Blood containing 15 % of parasitized RBCs was obtained from previously infected mice and was kept as a frozen stock. Prior to starting the experiment, parasites were thawed and two mice were intraperitoneally injected to ensure the supply of infective parasites for infection. Four days post-infection, blood drops were obtained from the tip of the tail of donor mice at 5-10 % parasitemia. Blood was then re-suspended in sterile PBS 1× to adjust the volume of administration, and 0.1 mL of this solution carrying 10^5–10^6 parasitized RBCs was then intraperitoneally injected into each mouse of the study.

Treatments and follow up of the infection

DCA and 2DG were both purchased from Sigma-Aldrich and kept following manufacturer's recommendations in a clean dry place at room temperature. Doses of 100 mg/kg and 200 mg/kg of body weight were selected for DCA, and 1000 mg/kg and 2000 mg/kg for 2DG. Both drugs were daily prepared in PBS 1×. Chloroquine diphosphate (Sigma) was used as a positive control at 5 mg/kg dose. Chloroquine (CQ) was only prepared once and kept at 4 °C for the

whole experiment due to its proven stability. The concentrations of these drugs were adjusted to a final volume of 0.2 mL per mouse.

The whole experimental procedure was divided into two different assays, following a "4-day and 7-day suppressive test" (Chen et al. 1994) for low and high drug concentrations respectively, in order to determine the parasite clearing ability of these compounds when compared to CQ. Once per day, 0.2 mL of the corresponding drug was intraperitoneally administered to each mouse. The first administration took place three hours post-infection, and animals were treated for 3 more consecutive days in case of low drug concentration, and 6 more days for high doses. Survival, torpor, lethargy, bouffant fur, and clinical signs of anemia were daily monitored for eight days. Parasitemia was determined at day 4, 5, and 8 post-infection by counting the number of infected erythrocytes in 600 red blood cells (RBC) from methanol-fixed and Giemsa (4 %) stained thin blood smears with the help of the Plasmoscore 1.3 software (Burnet Institute) (Proudfoot et al. 2008). At day 8, all the surviving animals were sacrificed after being anesthetized (Figure 18).

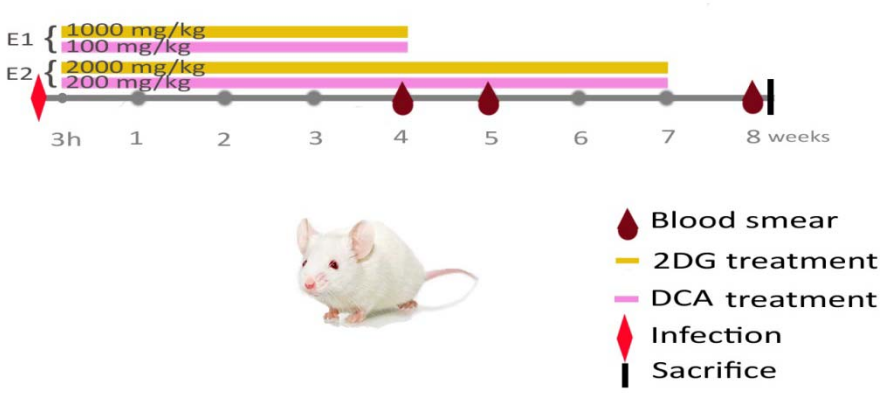


Figure 18: Graphic representation of the design of the two experimental assays (E1 and E2) performed in the *in vivo* model of murine malaria. Every assay had four experimental groups —untreated animals, chloroquine treated (both not shown in the figure), 2DG treated and DCA treated— of five animals each. Treatments lasted four days in E1, and 7 days in E2, and parasitemia was recorded at day 4, 5 and 8 for each assay. Acronyms: 2DG (2-deoxy-D-glucose), DCA (dichloroacetic acid), E1 (experiment 1) and E2 (experiment 2).

Statistical analysis

The statistical behavior of parasitemia results obtained in this work were analyzed at day 5 post-infection for both experimental approaches due to the lack of sufficient animals to perform statistical analysis by the last day of the experiment, although high doses of drugs were administered for longer periods. The software SPSS v15.0 (SPSS Inc.) was used to analyze the parasitemia readings by using ANOVA non-parametric statistical tests (Kruskal-Wallis), followed by the Mann-Whitney U test for single comparisons. Significant differences were considered when $P \leq 0.05$.

• Test of DCA, 3BP and MET on the *in vivo* model of American and African trypanosomiasis

Animals

Twenty-five female 5 week old Swiss Webster mice were used for each experiment. Animals were caged in groups of 5 mice each, depending on the future treatment: Sham-handled control (NC), DCA, 3BP, MET, and BDZ (for CD) or SUR (for the HAT model) as positive control drugs.

Parasites and infection

T. cruzi Brazil strain transfected with pTREX-luc were used for the *in vivo* Chagas disease model. Trypomastigotes for infection were collected from the supernatant of LLC-Mk2 (Kidney cells from rhesus monkey) infected cells grown in DMEM medium enriched with 2 % FBS and 1 % PSG. Mice were intraperitoneally administered with 10^5 of these infective forms.

For the HAT *in vivo* model, *T. brucei brucei* ANTAT1.1 strain previously transfected with Rluc-pHD309 plasmid was employed. These parasites were kindly provided by Dr. Nick Van Reet (Claes et al. 2009). Bloodstream form trypomastigotes cultured in HMI-9 medium were collected for infection and intraperitoneally inoculated at an adjusted concentration of 10^5 infective parasites.

Infection follow-up and drug treatment

Three days post-infection, basal luciferase values for each mouse were obtained by using an IVIS instrument (Xenogen, Alameda, CA). For that purpose, mice were anesthetized with 1.5 % isofluorane in air by using a gas anesthesia system, and then administered with 150 mg/kg of D-Luciferin Potassium-salt (Goldbio) in case of the CD model and 100 µL of a native coelenterazine solution for the HAT model, with both solutions prepared in PBS 1×. Mice were imaged 10 minutes later, analyzed with the software LivingImage (Xenogen), and then sorted into uniform groups of mean luminescence.

On the fourth day post-infection, treatments started and animals were intraperitoneally administered with the corresponding compound at established doses of 100 mg/kg of DCA, 5 mg/kg of 3BP, 125 mg/kg of MET and 15 mg/kg/day of BZD —for the CD model— or 40 mg/kg/day of SUR —for the HAT rodent model— for 5 days, respectively. Untreated control animals (C) received the same manipulation as the rest of animals and were injected with vehicle solution composed of 2 % of methylcellulose + 0.5 % Tween 80. Treatments were prepared daily and administered for 5 days. Once treatments were finished (on day 9 after infection), luciferase readings were recorded again as previously described (Figure 19). Final results are expressed as Relative Light Units (RLU).

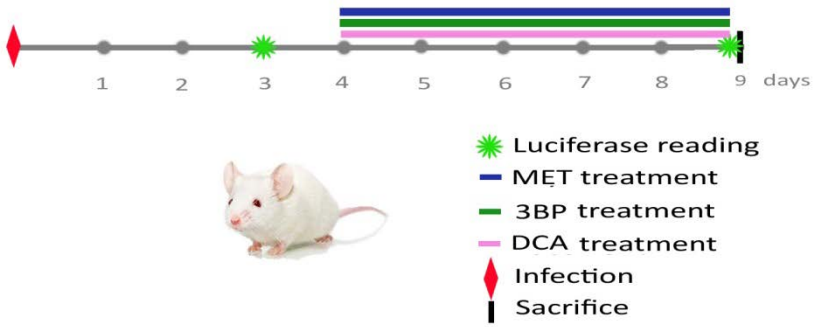


Figure 19: Graphic representation of the design of the experimental assays performed in the rodent model of HAT and CD. Every assay had five experimental groups —sham-treated animals, treated with control drug (not shown in the figure), treated with 3BP, treated with MET, and treated with DCA— of five animals each. Animals were infected with luciferase-transfected parasites (*T. cruzi Brasil* or *T. brucei brucei*). Treatments started 4 days post-infection and luciferase readings were recorded after 5 days of treatment. Acronyms: 3BP (3-bromopyruvic acid), DCA (dichloroacetic acid), and MET (metformin).

Statistical analysis

RLU Data were analyzed by using ANOVA non-parametric statistical tests (Kruskal-Wallis) and then the Mann-Whitney U test for single comparisons with the aid of SPSS v15.0 software (SPSS Inc). Significant differences were considered when $P \leq 0.05$.

• Test of DCA and 3BP on an *in vivo* model of VL

Animals

To perform this study, 22 male 7-weeks old Syrian golden hamsters (*Mesocricetus auratus*) of the RjHAN-AURA strain were obtained from *Centre d' Elevage René Janvier* (Le Genest-Saint-Isle, France). Animals were individually settled in plastic micro-filter cages, and food and water were provided *ad libitum.* The whole procedure was developed under bio-safety level 2 (BSL2) conditions at the *Servei d'Estabulari of Universitat Autònoma de Barcelona* (Bellaterra (Barcelona), Spain) and was approved by the *Universitat Autònoma* de Barcelona Animal Care Committee following the animal protection regulations according to the Directive 2010/63/UE. After a week post-arrival adaptation period, animals were randomly segregated into 4 different groups: two of them for drug treatment (DCA and 3BP groups) (n = 6 each), one vehicle sham-control (Control group) (n = 6), and one infected but untreated group aimed at controlling the development of the infection (IC group) (n = 4).

Parasites and infection

The *L. infantum* strain MCAN/ES92/BCN83/MON-1 was kindly provided by Dr. Portús, Universitat de Barcelona, Spain. This strain was firstly isolated from a naturally infected dog that received no previous treatment, and parasites were passaged in hamsters to retain their full virulence. Promastigotes were extracted from spleen samples and kept as a frozen stock. Promastigotes for infection were thawed and cultured at 26 °C in RPMI 1640 medium (Gibco) supplemented with 15 % heat-inactivated fetal calf serum (Gibco), 2 % HEPES 1 M (Gibco) and 1 % of total volume of 10000 U/mL penicillin, and 10000 µg/mL streptomycin (Gibco), and passages were performed once per week. The inoculum for infection was obtained from a 6-day old stationary culture, with the volume adjusted in physiological saline solution to 1×10^7

metacyclic promastigotes per 1 mL. All hamsters were infected through the intraperitoneal route.

Drug preparation and experimental procedure

DCA and 3BP were purchased from Sigma-Aldrich and kept under the conditions specified by the manufacturer. DCA and 3BP were prepared daily in plain RPMI 1640 (Gibco) to a concentration of 1.5 mg/mL and 15 mg/mL, respectively. Then, solutions were adjusted to a pH of 7.0 and filtered prior to administration. Treatment started at 11 weeks post-infection once IC animals confirmed the progression of infection. For that purpose, animals were weighed and then treated. Hamsters belonging to the 3BP group were intraperitoneally treated with 10 mg/kg 3BP for 7 consecutive days, while hamsters of the DCA group received 100 mg/kg DCA for 15 consecutive days, as reviewed in literature (Schaefer et al. 2012; Rooke et al. 2014) (Figure 20). The sham-control group was handled under same conditions as the other groups and was administered with plain RPMI medium.

One day after the last dose of treatment, blood samples were obtained by intracardiac puncture under general anesthesia, and right after, hamsters were euthanized in a CO_2 chamber. Tissue samples from spleen, liver, sub-mandibular lymph nodes and skin, were aseptically obtained and kept in formaldehyde, frozen, or in RNA later (Ambion) and stored at −20 °C. Spleen and liver were also measured and weighed.

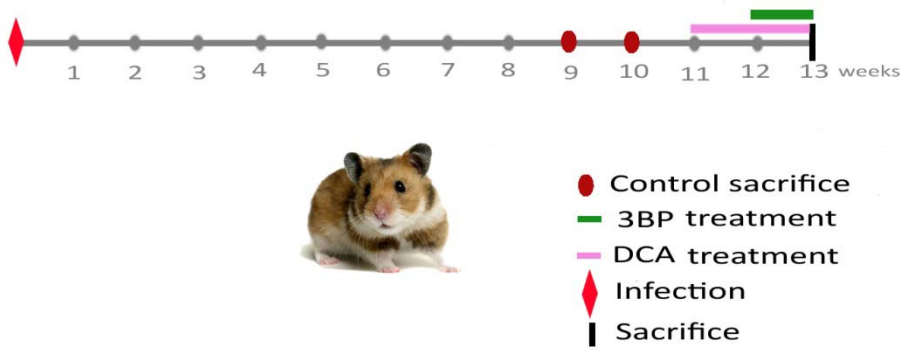


Figure 20: Graphic representation of the design of the experimental assay performed in the hamster model of VL. Three experimental groups —sham control group (not shown in the figure), treated with 3BP, and treated with DCA — of five animals each. Hamsters were infected with *L. infantum* and treated according to their corresponding group. Acronyms: 3BP (3-bromopyruvic acid) and DCA (dichloroacetic acid).

Total hamster IgG Anti-*Leishmania Infantum*

Blood samples were collected in tubes without anticoagulant and were centrifuged at 3000 rpm for 15 min to obtain serum. Sera were analyzed for the presence of anti-*Leishmania* IgG antibodies using the previously described ELISA technique (Todolí et al. 2012). Briefly, plates were coated with 2 µg per well of Crude Total *Leishmania* Antigen (CTLA) and sequentially incubated with sera and goat anti-hamster IgG-HRPO (ABD Serotech). Working dilutions were 1/400:1/1000 for sera dilution:IgG-HRP dilution. Absorbance values were read at 450 nm in the automatic microELISA reader Anthos 2001 (Anthos Labtec Instruments). Results were expressed in ELISA units (EU), which referred to a known positive serum used as a calibrator, and arbitrarily set to 1 EU. Cut-off value (mean + 3SD for 10 non-infected hamsters) was established at 0.047 OD.

The alternative activation of macrophages: arginase activity measurement.

To determine the arginase enzymatic activity and therefore the alternative activation of macrophages, we followed the protocol described by Corraliza et al. 1994 (Corraliza et al. 1994). A standard curve was constructed with two-fold serial dilutions of an established urea concentration. The arginase activity of our samples were determined as the amount of L-arginine transformation into urea in the presence of 1-phenyl-1,2-propanodione-2-oxime (ISPF). Obtained ODs of urea from our samples were recorded and interpolated in the standard curve with the GraphPadPrism 5 software and results are given as units of arginase activity (µg of urea resulting of L-arginine catalysis).

Absolute parasite quantification

Total genomic DNA was obtained from 50 mg of spleen, liver, lymph node, and skin tissue samples using the High Pure Template Preparation Kit (Roche), and was then measured by using a NanoDrop-2000 Spectrophotometer (Isogen Life science). All DNA samples were kept at −20°C until the analysis was performed. Kinetoplastid minicircle DNA sequences from *L. infantum* were used as specific primers to quantify parasite burdens, as previously described by Francino et al 2006 (Francino et al. 2006) (Table 7). Samples were run in triplicates in a Step One Plus Real Time PCR System (Applied Biosystems Laboratories) following a thermal cycling profile consisting of 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1

min. Resulting data were analyzed by using the StepOne Software v2.3. The number of parasites per µg of analyzed DNA was calculated by interpolation of counts obtained on a standard curve created with serial 1:10 dilutions of DNA from 1×10^7 promastigotes of a *L. infantum* culture.

Table 7: Sequence of primers and probe used for parasite detection.

Gene	Primer sequence	
Kinetoplastid	F	5'-AAC TTT TCT GGT CCT CCG GGT AG-3'
minicircle	R	5'-ACC CCCA GTT TCC CGCC-3'
DNA	P	FAM-5'-AAA AAT GGG TGC AGA AAT-3'- MGB

Acronyms: F, R and P mean Forward primer, Reverse primer and Probe, respectively. Data extracted from (Francino et al. 2006)

Cytokine expression analysis

Cytokine expression in spleen and liver tissues was studied. Samples of these tissues maintained in RNA later were homogenized with Tris Reagent and kept frozen till RNA extraction. Total RNA was extracted by using the RiboPure kit (Ambion) following manufacturer's instructions and samples were then treated with DNases TURBO-DNA free Kit (Ambion). The resulting RNA was measured with a NanoDrop-2000 Spectrophotometer (Isogen Life science) and the purity of these RNA samples was assessed by performing a qPCR to discard the presence of DNA. Clean RNA samples were stored at −80 °C till analyzed. Levels of IFN-γ, TNF-α, IL-4, IL-10, and TGF-β expression in liver and spleen were determined by using primers and FAM-labelled probes, in addition to γ-actin primers and its VIC-labelled probe for housekeeping control as described by Espitia et al. (Espitia et al. 2010) (table 8). Primers and probes were bought from Sigma Genomics. Retrotranscription and amplification of each sample was carried out in duplicate using TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems, Roche) with the aid of Applied Biosystems 7500 FAST PCR instrument and the Applied Biosystems 7500 Software V20.6. The thermal cycling profile was 48 °C for 30 min for retrotranscription, followed by 45 cycles of 95 °C for 10 min, 95 °C for 15 s, and 60 °C for 1 min. Cytokine mRNA expression levels were calculated by relative analysis using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Results are expressed as ×-fold more expression than the calibrator sample, which is based on the mean values of infected but untreated animals.

Table 8: Sequences of primers and probes used for expressed cytokines detection.

Gene		Primer sequence
γ-actin	F	5'-ACA GAG AGA AGA TGA CGC AGA TAA TG-3'
	R	5'-GCC TGA ATG GCC ACG TAC A-3'
	P	5'-VIC - TTG AAA CCT TCA ACA CCC CAG CC-(TAMRA)-3'
IL-10	F	5'-GGT TGC CAA ACC TTA TCA GAA ATG-3'
	R	5'-TTC ACC TGT TCC ACA GCC TTG-3'
	P	5'-(6FAM) TGC AGC GCT GTC ATC GAT TTC TCC C-(TAMRA)-3'
IFN-γ	F	5'-TGT TGC TCT GCC TCA CTC AGG-3'
	R	5'-AAG ACG AGG TCC CCT CCA TTC-3'
	P	5'-(6FAM) TGG CTG CTA CTG CCA GGG CAC ACT C-(TAMRA)-3'
IL-4	F	5'-ACA GAA AAA GGG ACA CCA TGC A-3'
	R	5'-GAA GCC CTG CAG ATG AGG TCT-3'
	P	5'-(6FAM) AGA CGC CCT TTC AGC AAG GAA GAA CTC C-(TAMRA)-3'
TGF-β	F	5'-GGC TAC CAC GCC AAC TTC TG-3'
	R	5'-GAG GGC AAG GAC CTT ACT GTA CTG-3'
	P	5'-(6FAM)-CCC TGT CCC TAC ATT TGG AGC CTG GA-(TAMRA)-3'
TFN-α	F	5'-TGA GCC ATC GTG CCA ATG-3'
	R	5'-AGC CCG TCT GCT GGT ATC AC-3'
	P	5'-(6FAM)-CGG CAT GTC TCT CAA AGA CAA CCA G-(TAMRA)-3'

Acronyms: F, R and P mean Forward primer, Reverse primer and Probe, respectively. Data extracted from (Espitia et al. 2010)

Histopathological studies

Samples from viscera and skin were collected to evaluate the histopathological changes. The tissue fragments from spleen, liver, lymph nodes, kidney, gut, and skin were collected, fixed in formaldehyde 10 % at pH 7.2, and processed by the usual techniques for optical microscopy. Semi-qualitative analysis was performed in order to evaluate the main histological findings observed in the different tissues, and the parameters were scored as (0) negative, (1) mild, (2) moderate, and (3) intense for each animal.

Statistical analysis

Data were analyzed by using non-parametric statistical tests (Kruskal-Wallis) and then by Mann-Whitney U test for single comparisons with the aid of SPSS v15.0 software (SPSS Inc). In

case of categorical ranked data —as is the case of the semi-quantitative study of the histology sections— the previously mentioned non-parametrical statistical tests were also used, assuming that the rating scale has an underlying continuity. The results were considered statistically significant when $P \leq 0.05$ (Bolton and Bon 2010).

RESULTS

- **Test of DCA and 2DG on the *in vivo* model of murine malaria**

Evolution of parasitemia score

Percentage of infected erythrocytes of control mice and mice treated with DCA 100 mg/kg and 1 g/kg 2DG showed a paralleled increase in parasite burden until the fifth day post-infection, with no significant differences between treated and control groups (DCA_{100} vs C: P= 0.999; $2DG_{1000}$ vs C: P= 0.999; $2DG_{1000}$ vs DCA_{100}: P= 0.841). From then on, a steep steady increase in parasitemia was recorded, and values corresponding to surviving mice given 100 mg/kg DCA and 1 g/kg 2DG reached higher levels than those obtained from untreated mice. Following the same pattern, animals given higher doses of these compounds (200 mg/kg DCA and 2 g/kg 2DG) shared similar parasitemia values with the untreated group until day 5 post-infection, not showing significant differences between them (DCA_{200} vs C P= 0.999; $2DG_{2000}$ vs C P= 0.421; $2DG_{2000}$ vs DCA_{200} P= 0.548). In contrast, at day 8 post-infection surviving animals showed parasitemia levels slightly lower than the control group. Animals that were treated with chloroquine showed no parasites from the first dose till the end of the experiment (DCA_{100} vs CQ: P= 0.008; DCA_{200} vs CQ: P= 0.008; $2DG_{1000}$ vs CQ: P= 0.008; $2DG_{2000}$ vs CQ: P= 0.008) (Figure 21). Images of stained blood smears are included (Figure 23).

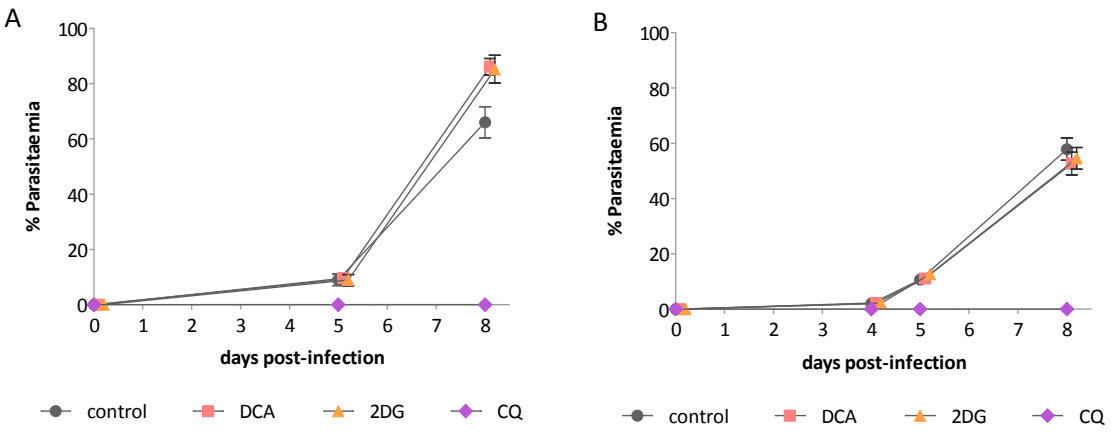


Figure 21: Percentage of infected erythrocytes from mice treated with DCA (100 mg/kg) and 2DG (1g/kg) (A), and DCA (200 mg/kg) and 2DG (2g/kg) (B). The blood parasite level of infected but untreated controls and CQ (5 mg/kg) treated are also shown. Acronyms: DCA (dichloroacetic acid), 2DG (2-deoxy-D-glucose), and CQ (chloroquine).

Survival rates, symptoms and treatment tolerance

No increase in life span was detected after the administration of high or lower doses of DCA or 2DG. Mice receiving low doses of 2DG started to die one day before the infected animals that were left untreated. Pale skin (ears, hands and feet) and apathy are anemia-associated symptoms caused by murine malaria that were detected, in addition to bouffant fur, in mice treated with DCA and 2DG —independently of the administered dose— and the untreated group from day 7 post-infection. Two mice receiving higher doses of 2DG also suffered from torpor and uncontrollable shivering, and another mouse from this group died on the same day. These three animals had red-colored urine compatible with hemoglobinuria. No clinical symptoms compatible with malaria or adverse drug effects were detected in the chloroquine-treated group of mice, with all animals belonging to this group remaining alive by the end of the study (Figure 22). Animals alive at the end of the study were sacrificed to avoid suffering.

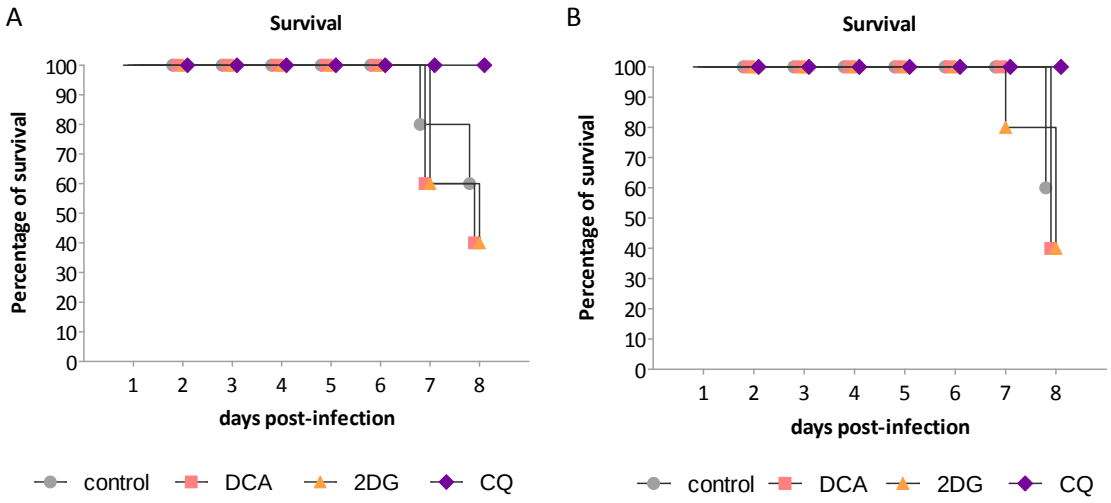


Figure 22: Survival rates of *P. yoelii*-infected mice when treated with DCA (100 mg/kg) and 2DG (1 g/kg) (A); and DCA (200 mg/kg) and 2DG (2 g/kg) (B) in comparison with CQ and untreated animals. Acronyms: DCA (dichloroacetic acid), 2DG (2-deoxy-D-glucose), and CQ (chloroquine).

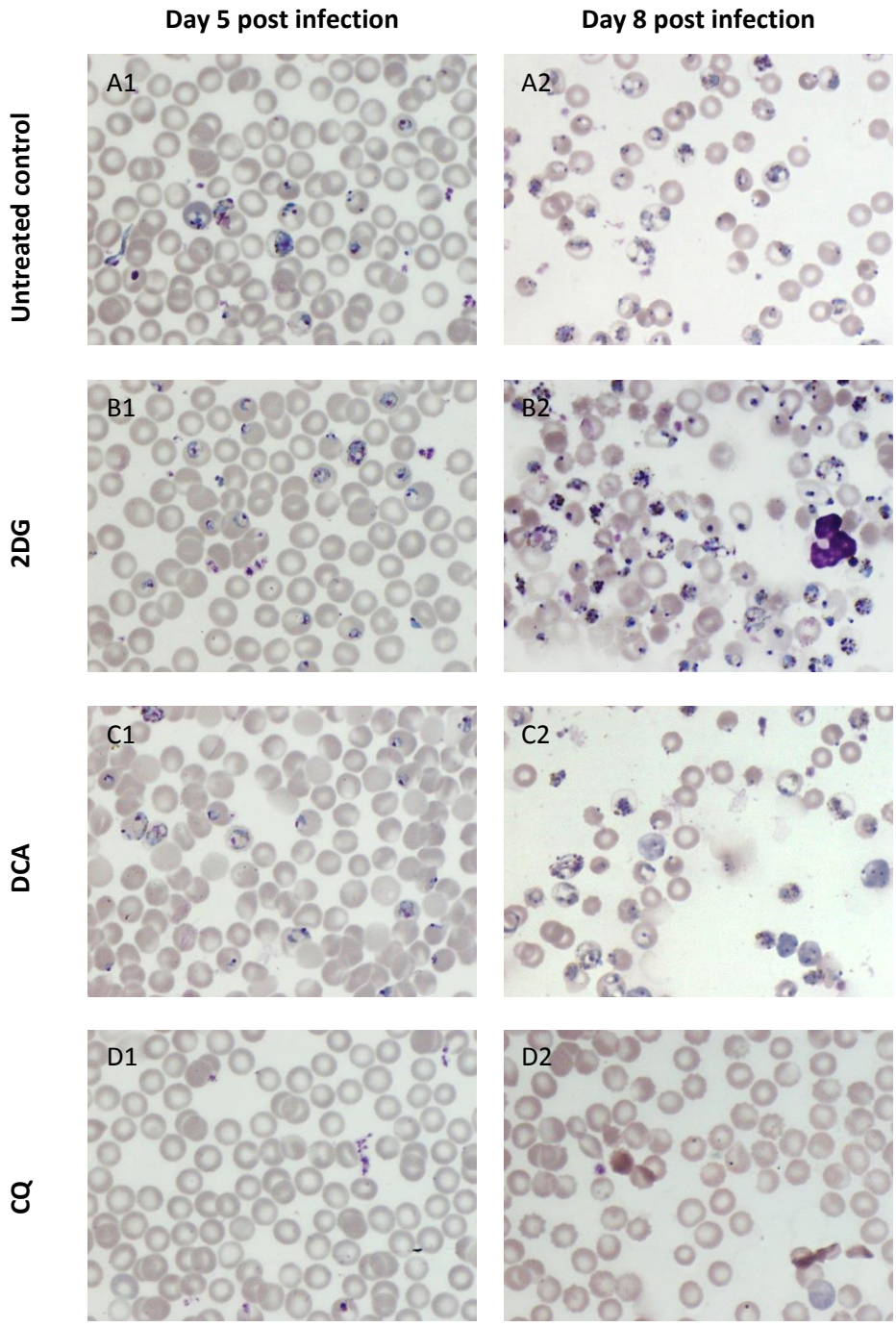


Figure 23: Giemsa stained thin blood smears. (A) Untreated animals, (B) 2DG treated animals (2 g/kg), (C) DCA treated animals (0.2 g/kg), and (D) CQ treated animals at days 5 (1) and 8 (2) post-infection. Except for CQ treated animals, parasites in different stages of development can be observed at 5 days post-infection resulting in lower parasite levels and lower density of RBCs by day 8 post infection. Acronyms: DCA (dichloroacetic acid), 2DG (2-deoxy-D-glucose), and CQ (chloroquine).

- **Test of DCA, 3BP and MET on the *in vivo* model of American and African trypanosomiasis.**

Evolution of the infection and disease

RLU obtained from DCA or 3BP treated animals were similar to those from animals administered with the vehicle alone (untreated control), with no significant differences observed between them in the rodent models of African (DCA vs C $P = 0.556$; 3BP vs C $P = 0.114$; DCA vs 3BP $P = 0.413$) and American trypanosomiasis (DCA vs C $P = 0.999$; 3BP vs C $P = 0.841$; DCA vs 3BP $P = 0.999$). No comparable data for parasite burden differences were possible to measure in the MET treated animal groups, as most of mice administered with this compound for both American and African trypanosomiasis models died within the first days of treatment. Control drugs SUR and BZD, completely abrogated parasite multiplication in the animal model of their corresponding disease, as no increase in luminescence was detected (Figure 24). In spite of the increasing parasite burden detected in mice that were not treated with SUR or BZD, no clinical manifestations of the disease were observed during this trial.

Treatment tolerance, symptoms and survival rates

No signs of pain or adverse reactions were observed immediately after the administration of the compounds or vehicles during these studies.

Apart from the apparent MET toxicity, only two mice from the *in vivo* model of CD — one belonging to the 3BP-treated group and another from the group that was administered only with the vehicle— died before the end of the experiment, with no apparent symptoms of disease or suffering (Figure 25).

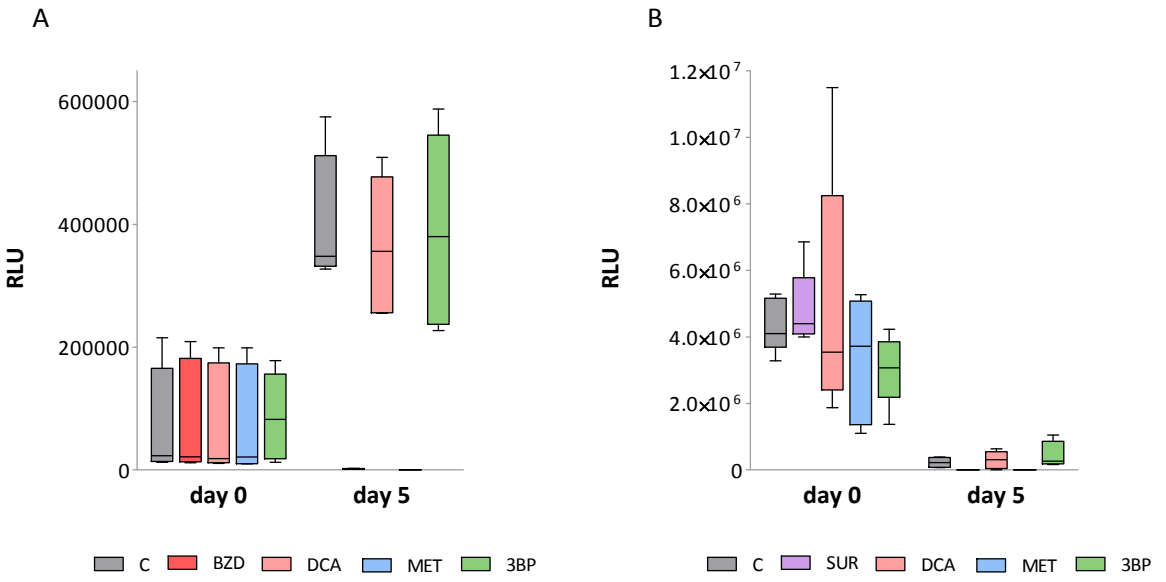


Figure 24: RLU showing the evolution of *T. cruzi Brasil* (A) and *T. brucei brucei* (B) infections after 5 days of treatment with DCA, MET or 3BP in comparison with control drugs. Acronyms: DCA (dichloroacetic acid), 3BP (3-bromopyruvic acid), MET (metformin), SUR (suramin) and BZD (benznidazole).

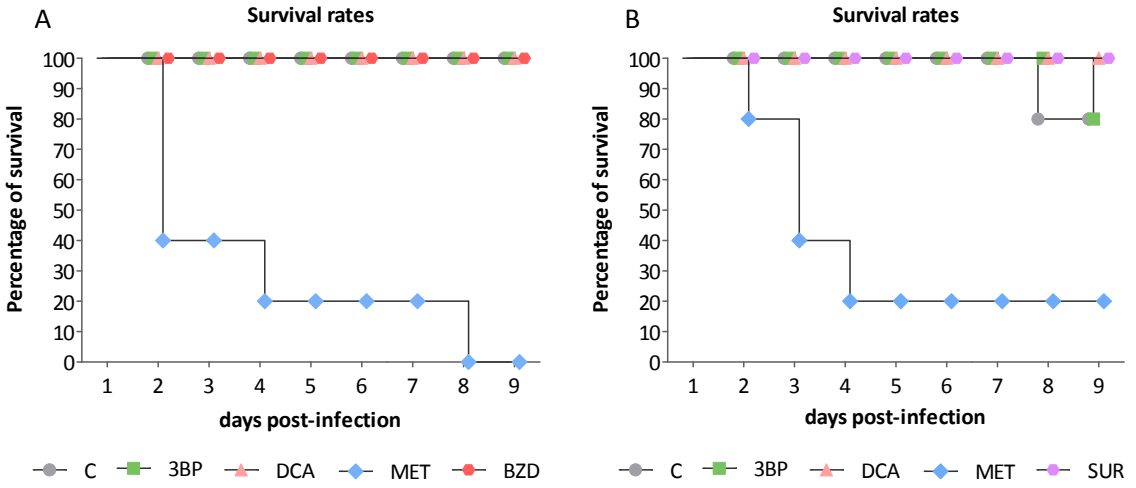


Figure 25: Survival rates of mice infected with *T. cruzi Brasil* (A) and *T. brucei brucei* (B) after 5 consecutive days of treatment with selected bioenergetics modulators. Acronyms: DCA (dichloroacetic acid), 3BP (3-bromopyruvic acid), MET (metformin), SUR (suramin) and BZD (benznidazole).

- **Test of DCA and 3BP on an *in vivo* model of VL**

Treatment tolerance

Three animals in this study (one belonging to the DCA group and other two from the 3BP group) presented with adverse events such as apathy, apparent diarrhea, and a distended area in cranial abdomen. However, all animals had already completed the treatment protocol, and therefore, specimens belonging to these animals were not discarded.

When sacrificed after receiving the last dose of treatment, all animals belonging to the 3BP group had swollen intestine areas with liquid content in its interior, being compatible with peritoneal irritation. These observations confirm the results of Barnard (Barnard et al. 1993). Although the doses administered in this study were taken from literature and no adverse effect was described in these works —even when authors used higher doses (Gargaglioni et al. 2003; Schaefer et al. 2012)—, symptoms recorded in our laboratory suggest that 3BP administration cause pain and peritoneal irritation, which that can be aggravated by repeated administrations (Turner, Brabb, et al. 2011).

Evolution of the infection and disease in treated hamsters

No significant differences in size or weight for spleen and liver were found between hamsters treated with DCA and the untreated group ($P = 0.628$). Conversely, animals that received 3BP showed a significantly lower spleen size and spleen and liver weight than the control group ($P = 0.012$ and $P = 0.006$, respectively). Spleen and liver enlargement are recurrently described as typical signs of VL progression, as they reflect the breakdown of tissue architecture due to parasite multiplication, granuloma formation, loss of specific cell populations, and other inflammatory processes (Kaye et al. 2004). No weight loss was observed in the hamsters (a characteristic symptom of VL) while this experiment lasted.

Parasite DNA was detected in all hamsters by the day of sacrifice, as shown by qPCR analysis, reflecting effective infection. When parasite burdens were analyzed, no significant differences were detected for any of the analyzed target tissues between experimental groups. Overall, higher parasite burdens were detected in spleen and liver, followed by lymph nodes and skin in all studied groups. Although results were variable even between animals belonging to the

same group, a tendency was observed in 3BP-treated animals to have a higher number of parasites than other groups in spleen, liver and lymph nodes (Figure 26).

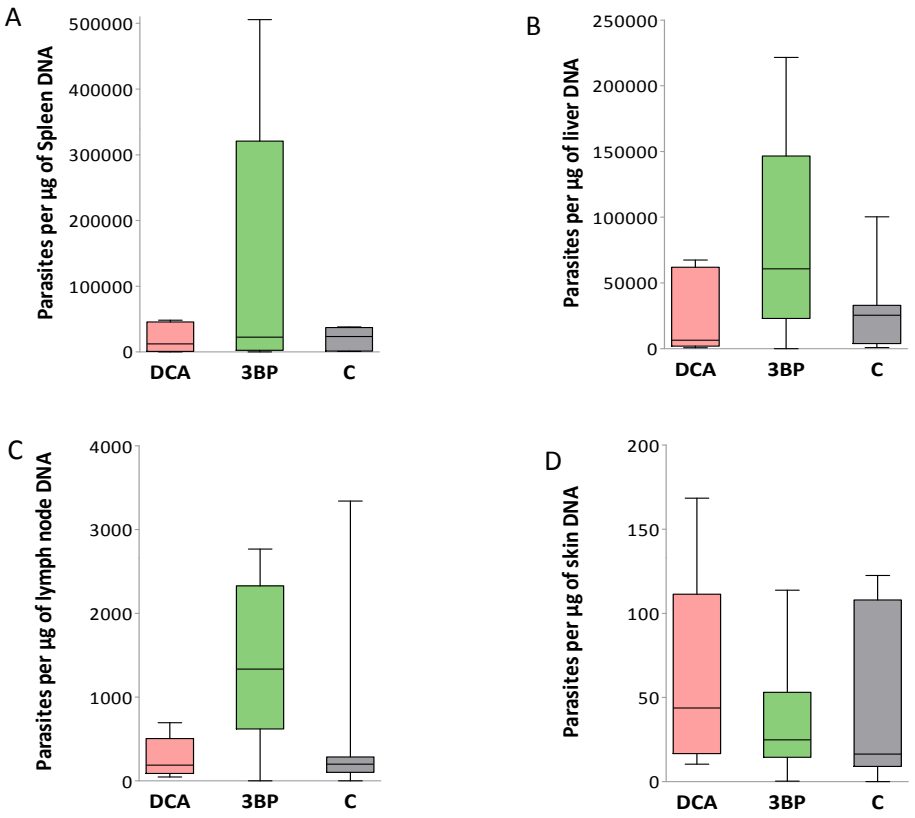


Figure 26: Comparison of absolute numbers of parasites detected in spleen (A), liver (B), lymph node (C) and skin (D) of *L. infantum*-infected hamsters after receiving DCA, 3BP or vehicle solution. Acronyms: 3BP (3-bromopyruvic acid), DCA (dichloroacetic acid), and C (control).

Analysis of the immune response:

Arginase activity and IgG production as immune related indicators

The arginase activity is a helpful parameter to evaluate alternative activation of macrophages, which is associated with an impaired control of the intracellular multiplication of *Leishmania*. The quantity of urea from the *ex vivo* L-arginine modification by spleen cells indicates there is higher arginase activity in untreated animals. Hamsters receiving DCA (P = 0.001) and 3BP (P = 0.006) showed significantly lower arginase activity than the control group. Arginase activity values corresponding to samples from the 3BP group were lower than the DCA group (P = 0.010).

By the day of sacrifice, all animals except one hamster of the 3BP group, showed positive levels of *Leishmania*-specific IgG, that was significantly lower for 3BP-treated animals when compared to DCA ($P = 0.010$) and C ($P = 0.042$) groups (Figure 27).

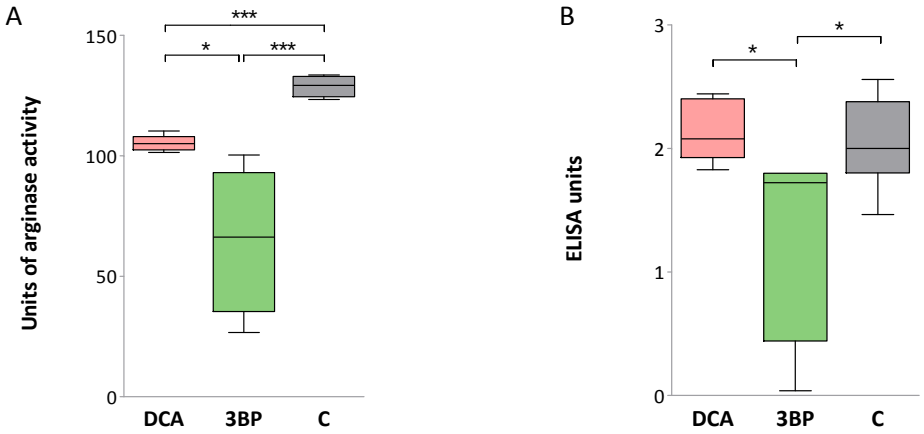


Figure 27: Arginase activity (A) and serology values (B) detected in infected hamsters after DCA, 3BP or diluent administration protocols. Acronyms: 3BP (3-bromopyruvic acid), DCA (dichloroacetic acid), and C (control) (* $P < 0.05$, *** $P < 0.001$).

Cytokine production

Expression of pro- and counter-inflammatory cytokines studied in this work —IFN-γ, TNF-α, TGF-β, IL-10 and IL-4— was detected by the day of sacrifice in spleen and liver samples. As a general pattern, treated groups showed lower levels of pro- and counter-inflammatory studied cytokines than the control group (Table 9).

In case of IFN-γ, a significant reduction in the expression of this cytokine was detected for spleen samples when comparing DCA ($P = 0.001$) and 3BP ($P = 0.003$) to untreated animals. Conversely, no statistical differences were appreciated in liver (DCA vs C, $P = 1$; 3BP vs C, $P =$ 0,106). TNF-α expression follows the same pattern for spleen and liver samples. Animals treated with 3BP ($P = 0.003$) and DCA ($P = 0.051$) showed lower values of TNF-α expression when compared to the C group in spleen. These differences were also extended to liver samples (DCA vs C, $P = 0.051$; 3BP vs C, $P = 0.003$).

In the case of counter-inflammatory cytokines, IL-10 production showed statistically lower mRNA levels in spleen from DCA treated animals ($P = 0.001$). IL-4 production follows the same

pattern for spleen and liver samples, where 3BP and DCA animals showed lower expression in spleen (DCA vs C, *P* = 0.051; 3BP vs C, *P* = 0.006) and liver (DCA vs C, *P* = 0.003; 3BP vs C, *P* =0.003). Spleen TGF-β expression was variable and no statistical differences were detected for the three experimental groups. Conversely, there were lower levels of this cytokine in liver for treated groups that was significant for 3BP when compared to the control group (*P* = 0.003).

Table 9: Folds of median values (Q1-Q3) in comparison to median values of the C group that were previously adjusted to 1, and *P*-values for the transcription of target cytokines within spleen and liver samples of *L. infantum*-infected hamsters administered with 3BP, DCA, or vehicle solution.

TISSUE	GENE	C	DCA	3BP	DCA VS C	3BP VS C	3BP VS DCA
SPLEEN	***IFN-γ***	1	0.064 (0.001-0.139)	0.0005 (0.000-0.001)	***P* = 0.001***	***P* = 0.003***	*P* = 0.177
	TNF-α	1	0.672 (0.259-0.809)	0.270 (0.167-0.272)	*P* = 0.051	***P* = 0.003***	*P* = 0.329
	IL-10	1	0.266 (0.056-0.501)	0.017 (0.007-2.427)	***P* = 0.001***	*P* = 0.639	*P* = 0.792
	IL-4	1	0.163 (0.110-0.611)	0.245 (0.054-0.482)	*P* = 0.051	***P* = 0.006***	*P* = 0.476
	TGF-β	1	1.084 (0.625-1.387)	0.939 (0.786-1.088)	*P* = 1.000	*P* = 0.237	*P* = 0.762
LIVER	***IFN-γ***	1	1.119 (0.882-1.825)	0.370 (0.022-0.703)	*P* = 1.000	*P* = 0.106	*P* = 0.177
	TNF-α	1	0.592 (0.369-0.858)	0.123 (0.100-0.148)	*P* = 0.051	***P* = 0.003***	*P* = 0.082
	IL-10	1	0.933 (0.826-17.016)	1.553 (1.527-91.434)	*P* = 0.366	*P* = 0.106	*P* = 0.429
	IL-4	1	0.056 (0.042-0.094)	0.086 (0.071-0.109)	***P* = 0.003***	***P* = 0.003***	*P* = 0.690
	TGF-β	1	0.671 (0.429-0.817)	0.379 (0.226-0.533)	*P* = 0.106	***P* = 0.003***	*P* = 0.548

Acronyms: C (control group), DCA (DCA-treated group), 3BP (3BP-treated group), IFN-γ (Interferon γ), TNF-α (Tumor necrosis factor α), TGF-β (Transforming growth factor β), IL-10 (Interleukin-10), and IL-4 (Interleukin-4) (* $P < 0.05$).

Histopathological analysis

The most frequent histopathological alterations detected in this study in infected hamsters are described below. For spleen and liver, both organs showed hyperplasia and hypertrophy of macrophages to a similar degree between experimental groups, in addition to the presence of granulomas formed by aggregates of mononuclear cells, which were found to be present in a similar number between all the experimental groups in spleen. In the case of liver samples, it was similar between the control and DCA group, but was observed to a lower extent in the 3BP group (*P* = 0.014). Mild activation of splenic white pulp follicles was also observed in the DCA and control group, with no significant difference between them. In liver, a mononuclear

inflammatory infiltrate with parasites was also detected in the portal area, which was significantly lower in the 3BP group when compared to IC (P = 0.005). As regards the lymph nodes, a mononuclear infiltrate was also detected in the para-cortical area in the same degree between the experimental groups. Hyperplasia and hypertrophy of macrophages in the medullar area was the main histological feature observed among the experimental groups. The cortical area showed follicular hypoplasia, especially in the DCA and control groups, but aspects of activation were also present in one animal belonging to the DCA group, and another of the 3BP group. Figure 28 shows the main histological features observed in spleen, liver, and LN. Kidney, gut, and skin did not show histological changes in any experimental group (data not shown).

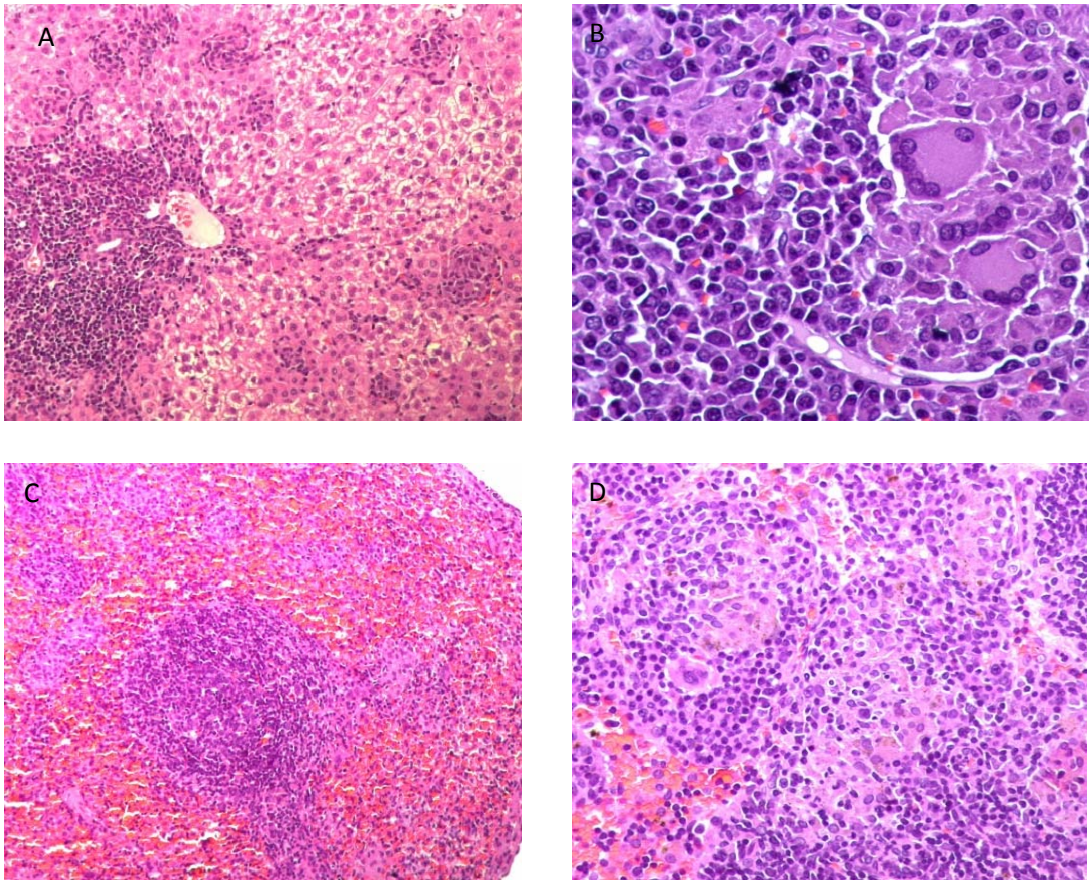


Figure 28: Histopathological findings observed in infected hamsters. (A) Liver (HE ×100) granulomas and mononuclear infiltrate in the peri-portal area. (B) Liver (HE ×200), hyperplasia and hypertrophy of Kupffer cells. (C) Spleen (HE ×40), hyperplasic lymphoid follicles of the white pulp and granulomas in the red pulp. (D) Lymph nodes (HE ×100), mononuclear infiltrate and hyperplasia and hypertrophy of macrophages in the medullar area.

SIR as a vaccine protection booster against visceral leishmaniasis

Summary of the experimental approach:

This chapter is destined to tackle the last objective of this work:

"To determine if the concomitant administration of sirolimus during the immunization with a DNA vaccine may protect against a posterior challenge with *L. infantum*"

In the search for a safe and efficient protective vaccine for human visceral leishmaniases, we used the sirolimus bioenergetic modulator (known for boosting immune-mediated protection against different disease models) to boost the effect of a DNA vaccine. We tried to potentiate the effect of a DNA vaccine designed in our laboratory that carried the *Leishmania* genes *LACK*, *TRYP*, *PAPLE22* and *KMPII*. For that purposes Syrian hamsters were treated with sirolimus concomitantly with the administration of our DNA vaccine. The parasite burden of different *Leishmania* target tissues and immune related parameters —immune markers expression, serology, or macrophage polarization indicators— were studied in animals receiving the boosted vaccine and compared with animals that received the vaccine alone and with infected but non-vaccinated control animals.

MATERIALS AND METHODS

Parasites

The *L. infantum* strain MCAN/ES/92/BCN83 (zymodeme MON-1) was kindly provided by Dr. Portús, Universitat de Barcelona, Spain. It was obtained from a naturally infected and untreated dog and maintained through hamster passages in order to retain its full virulence.

Promastigotes were cultured at 26 °C in R15 medium (RPMI 1640 medium (Gibco) supplemented with 15 % heat-inactivated fetal calf serum (Gibco), 2 % HEPES 1 M (Gibco) and 1 % of total volume of 10000 U/mL penicillin, and 10000 µg/mL streptomycin (Gibco)) and weekly passages were performed.

Metacyclic promastigotes for infection were obtained from a 6-day-old stationary culture, washed and maintained in PBS until the moment of inoculation. Hamsters were intraperitoneally injected with 1 mL of PBS containing 1×10^7 parasites.

Animals

Thirty male, 7 weeks-old, Syrian golden hamsters (*Mesocricetus auratus*, strain RjHAN-AURA) were obtained from Centre d'Elevage René Janvier (Le Genest-Saint-Isle, France). The animals were kept individually in plastic micro-filter cages under BSL2 conditions at the Servei d'Estabulari of Universitat Autònoma de Barcelona (Barcelona, Spain) and food and water were provided *ad libitum*. All procedures were approved by the Universitat Autònoma de Barcelona Animal Care Committee following the principles of animal protection according to the Directive 2010/63/UE. After an adaptation period of one week, the hamsters were randomly distributed into 3 experimental groups of 10 animals each: one treated and vaccinated group (S+V), one vaccinated group (V), and one infected non-vaccinated untreated control (C).

Sirolimus treatment

An oral solution of Rapamune 1 mg/mL (sirolimus) was purchased from Pfizer and kept under conditions specified by the manufacturer. Treatment doses of 0.075 mg/Kg in 500 µL of PBS 1×

were prepared daily under aseptic conditions (Araki et al. 2009). The S+V group first received a daily dose intraperitoneally from day −1 (one day prior to the first vaccination) for 6 consecutive weeks. The other two remaining groups of hamsters were handled under the same conditions, being administered with 1× PBS.

Vaccine preparation

Sequences of the elected genes —*TRYP*, *PAPLE22*, *LACK* and *KMPII*— were cloned individually into a pVAX 1 vector, and then over-expressed in *E. coli* cultures and purified as described elsewhere (Rodríguez-Cortés et al. 2007; Todolí et al. 2012). To ensure the presence and integrity of these sequences, 10 µg of each plasmid was digested for 3 h at 37 °C by using the Roche restriction enzymes referred below (Table 10) and DNA fragments were run in a 1 % agarose gel.

Table 10: Fragment lengths and restriction enzymes to ensure the presence and integrity of *LACK*, *PAPLE22*, *TRYP* and *KMPII* in the generated plasmids for naked DNA vaccination.

SEQUENCE	RESTRICTION ENZYME	FRAGMENT LENGTH
LACK	ECoRI	942 pb
PAPLE22	PstI and NotI	750 pb
TRYP	ECoRI	609 pb
KMPII	ECoRI	279 pb

Vaccine administration

Three doses of the vaccine containing 100 µg of each plasmid were administered intramuscularly into the hind limb, with two weeks of difference between doses. In order to maintain the same handling conditions as the other groups, the control group was injected with physiological saline solution (PSS) instead.

Infection

Two weeks after the administration of the last dose of the vaccine, all animals were intraperitoneally inoculated with 1 mL of PBS containing 1×10^7 *L. infantum* metacyclic promastigotes.

Animal weight and typical VL clinical signs in the rodent model —hair and weight loss, and cutaneous lesions— were evaluated every two weeks to ensure animal welfare.

In order to follow the development of the infection, the infected control group was euthanized 2 and 4 weeks after parasite inoculation, and the burden of parasites from spleen and liver were evaluated using a real time-PCR procedure (Figure 29).

Necropsy and sample collection

The remaining animals were euthanized 5 weeks after parasite inoculation (Figure 29). Hamsters were deeply anesthetized with a 100 µL intramuscular injection containing ketamine (42.8 mg/kg), acepromazine (1.4 mg/kg), and xylazine (9.5 mg/kg). An intracardiac puncture was performed to obtain 5 mL of blood sample from each animal, which were then euthanized in a CO_2 chamber. The abdominal cavity was then opened and organs removed. Samples of spleen, liver, sub-mandibular lymph nodes (LN) and skin were aseptically obtained and kept on ice to avoid tissue degradation.

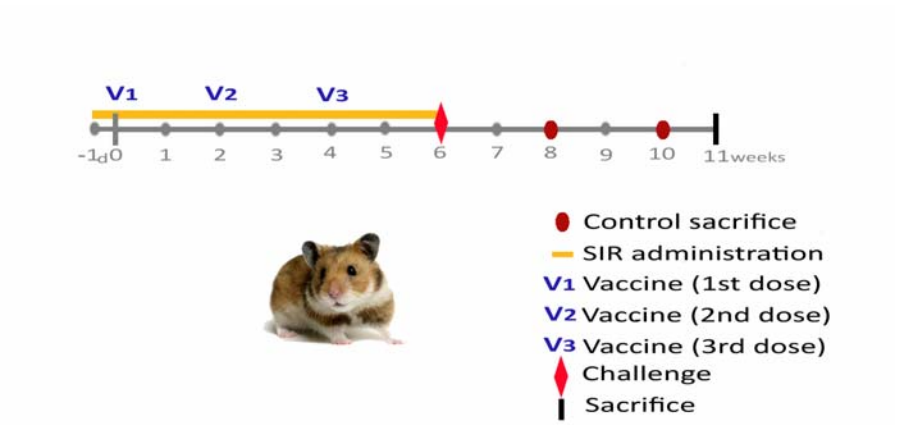


Figure 29: Graphic representation of the design of the experimental assay. Animals were divided into three experimental groups: vaccinated, vaccinated and SIR-treated, and control. Hamsters received sirolimus or a PBS solution daily from one day prior to first vaccination for 6 consecutive weeks. A total of 3 vaccine doses were administered, with a 2 week period between them. All animals were challenged two weeks after receiving last vaccination dose, and sacrificed 5 weeks later. Acronyms: V1 (first vaccine dose), V2 (second vaccine dose), and V3 (third vaccine dose).

Evaluation of humoral immune response: Total hamster IgG Anti-*L. infantum*

Blood samples were collected in hematology crystal tubes and spun at 3000 rpm for 15 min to obtain serum samples and were frozen till analyzed. ELISA plates (Costar high binding transparent flat bottom) were coated overnight at 4 °C with 2 µg per well of Crude Total *Leishmania* Antigen (CTLA) in 100 µL of carbonate-bicarbonate coating buffer (0.1 M NaCO3-H2CO3 pH 9.6). Samples were diluted 1:100 in 1 × PBS with 0.05 % Tween 20 and 1 % of skimmed milk powder (PBSTM) and serially diluted in the pre-coated plate. Plates were incubated at 37 °C in a humid atmosphere for 1 h and were then washed 3 times with PBST and once with PBS. Plates were then incubated for 1 h at 37 °C with goat anti-hamster IgG-HRPO (ABD Serotech) diluted 1:1000 in PBSTM. Antibody excess was removed with another cycle of washes as described above. Then, 100 µL of the peroxidase substrate Tetramethylbenzidine (TMB) (Sigma Aldrich) was added to each well, and the plates were left to develop for 5 min. The reaction was stopped by the addition of 50 µL of 1 M H_2SO_4, and absorbance was recorded at 450 nm (Anthos 2001 reader). The cut-off value was established at 0.047 OD, and was set as three times the standard deviation of the obtained optical densities (ODs) of 10 non-infected hamster serum samples.

The alternative activation of macrophages: arginase activity measurement

To determine the arginase enzymatic activity and therefore the alternative activation of macrophages, a standard curve was built with two fold serial dilutions of an established urea concentration. The arginase activity of the samples was determined as the amount of L-arginine transformation into urea in the presence of 1-phenyl-1,2-propanodione-2-oxime (ISPF) (Corraliza et al. 1994). Obtained ODs of urea from our samples were analyzed and interpolated in the standard curve with the GraphPadPrism 5 software and results are given as units of arginase activity (µg of urea resulting of L-arginine catalysis).

DNA extraction and real-time PCR absolute parasite quantification

Every step was performed following good laboratory practices to avoid DNA cross-contamination. Total genomic DNA was obtained from 50 mg of spleen, liver, skin and LN samples by using the High Pure Template Preparation Kit (Roche) following the mouse tail protocol provided in the manufacturer's guidelines. DNA concentration was measured by using

a NanoDrop-2000 Spectrophotometer (Isogen Life science). All DNA samples were kept at −20 °C until the qPCR was performed.

To specifically detect and quantify parasite burdens, primers targeting conserved DNA regions of the kinetoplast minicircle DNA from *L. infantum* and TaqMan-MGB probes were used as formerly described (Francino et al. 2006) (table 7). A standard curve was constructed from the total amount of DNA obtained from 1×10^7 promastigotes of an *L. infantum* culture by using serial dilutions from 10^3 to 10^{-3} parasites. Triplicates of 25 ng of DNA of each sample, a negative control, and standard curve were run in a Step One Plus Real Time PCR System (Applied Biosystems) following a thermal cycling profile consisting of 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 seconds, and 60 °C for 1 min. The resulting data were analyzed using the StepOne Software v2.3, and final results are given as number of parasites per µg of DNA.

Cytokine determination

Cytokine expression was studied in spleen tissue. Samples maintained in RNA later (Ambion) were homogenized with Tris Reagent (Ambion) and kept frozen until RNA extraction. Total RNA was extracted by using the RiboPure kit (Ambion) following the manufacturer's instructions, and samples were then treated with DNases TURBO-DNA free Kit (Ambion). Clean RNA samples were stored at −80 °C until analyzed. Retrotranscription was carried out by using High capacity cDNA Reverse transcription kit (Applied Biosystems) following a thermal profile of 25 °C for 10 min, 37 °C for 120 min, 95 °C for 5 min. Levels of IFN-γ, FoxP3, TNF-α, and TGF-β expression were determined in addition to RPL18 as a control gene for relative gene expression (Zivcec et al. 2011). To ensure amplification of cDNA sequences derived from retrotranscription of mRNA of interest, primers were designed including exon boundary sequences based on basic primer information extracted and modified from Espitia and Zivcec (Espitia et al. 2010; Zivcec et al. 2011) (table 11). Rat (*Rattus norvergicus*) sequences were used for exon-boundary areas determination, as hamster sequences are not available for ENSEMBL analysis (Ensembl 2017), and previous BLAST and CLUSTALW analysis confirmed sequence identity. Amplification of each sample was carried out in triplicate by using SYBR select RT-PCR Master Mix Reagents (Applied Biosystems) with the aid of Applied Biosystems 7900 HT FAST REAL-TIME PCR instrument and the SDS Software V2.3. The thermal cycling profile was 10 min at 50 °C, followed by 40 cycles of 95 °C for 10 min, 95 °C for 15 s, and 60 °C for 1 min. Melting

curves were included for every sample. Cytokine mRNA expression levels were calculated by relative analysis using the $2^{-\Delta\Delta CT}$ method using RPL18 as housekeeping gene and control group mean values for calibration (Livak and Schmittgen 2001). Results are expressed as ×-fold more expression than values of the C group.

Table 11: Sequences of primers and probes used for expressed cytokines detection.

Gene	Primer sequence	
RPL18	F	5'-GTC CCT GTC CCG GAT GATC-3'
	R	5'-GAC AGT CCC CAC AAC CACG-3'
IFN-γ	F	5'-CTA TGT CTG GCT GCT ACT GCCA-3'
	R	5'-TTC ACG ACA TCT AAG CTA CTT GAG TTAA-3'
TNF-α	F	5'-CCA GAC ACT CAC ACT CAG ATC ATC TT-3'
	R	5'-CAC TTG GTG GTT TGC TAC AAC GT-3'
TGF-β	F	5'-ACA GAA AAA GGG ACA CCA TGC A-3'
	R	5'-GAA GCC CTG CAG ATG AGG TCT-3'
FoxP3	F	5'-CAA GTG GCC TGG TTG TGA GA-3'
	R	5'-TGA TCT GCT TGG CAG TGCTT-3'

Acronyms: F and R mean Forward primer and Reverse primer, respectively. Data extracted and modified from Espitia and Zivcec (Espitia et al. 2010; Zivcec et al. 2011)

Statistical analysis

Exploratory data analysis and inference was carried out by using R statistical software v3.2.5 (The R Foundation for Statistical Computing, 2008). Inference was performed using functions included in the WRS2 package v0.4-0 (Mair, P., Schoenbrodt, F., & Wilcox 2015). Robust statistical methods and bootstrapping were used due to data violation of assumptions for classical statistical methods and, also, to their higher precision and statistical power in comparison to non-parametric tests (Wilcox 2012).

RESULTS

Presence of DNA sequences

Agarose gel confirmed the presence and the correct length of the *Leishmania* gene sequences inserted in the pVAX vector. The observed bands were in accordance with the expected size for sequences of *LACK* (942 pb), *PAPLE22* (750 pb), *TRYP* (609 pb), and *KMPII* (279 pb) (Figure 30).

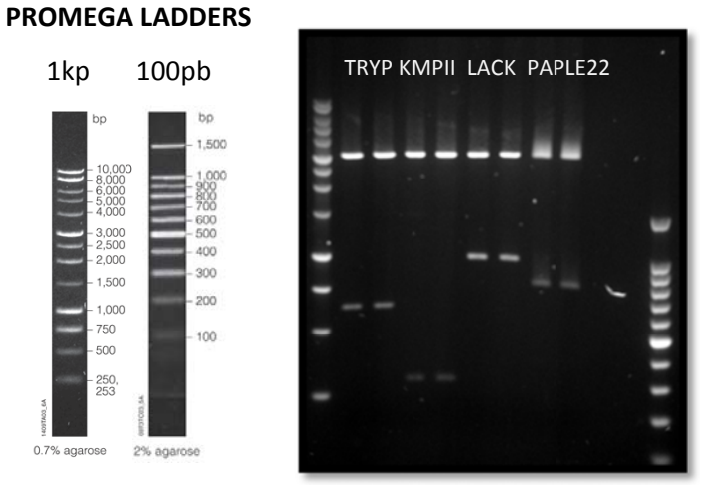


Figure 30: Agarose gel (1 %) showing the fragments of known immunogenic *Leishmania* genes after digestion of the plasmid vectors used in this study as a DNA vaccine.

Sirolimus and vaccine tolerance

Hamsters, regardless of which group they belonged, did not show any leishmaniasis-related symptoms and no animal needed to be euthanized during this experiment. No significant differences in final weight were found between groups at the end of the study.

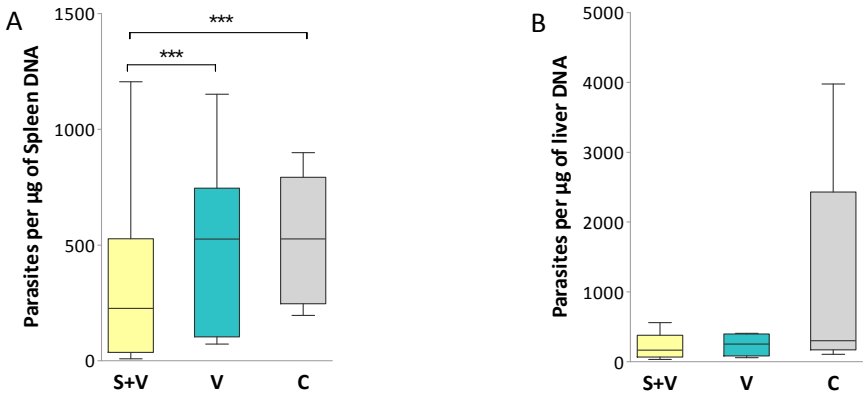
Two weeks after the start of the experimental procedure, 7 animals showed bleeding wounds on the tail. Five of them belonged to the S+V group, whereas the other two belonged either to the control or to the V group. Three of these S+V animals did not achieve complete resolution of the lesion by the end of the procedure. No adverse reactions were observed after DNA vaccination.

Parasitological protection

Animals from the V group showed a general reduction in the number of parasites compared to the control group, which was statistically significant for skin (*P* = 0.0004) and LN (*P* = 0.0452). Non-significant differences were observed for liver (*P* = 0.1072) and spleen (*P* = 0.8372).

Animals that received SIR during the administration of the vaccine also had a statistically reduced amount of parasites in LN (*P* = 0.0489) and skin (*P* = 0.0400), which was also expanded to a significant protection of spleen (*P* = 0.0004) with parasite burdens concurrently lower than the V group (*P* = 0.0080). Although non-statistically significant (*P* = 0.0598), there is also a noticeable tendency of a parasite burden reduction in the liver of hamsters of the S+V group compared to the C group (Figure 31).

It is worth noting that there were animals in both vaccinated groups —V and S+V— with no parasites in skin samples. When comparing S+V and C, no parasites were found in 6/10 hamsters versus 1/6 in control animals, and a 78 % reduction in mean values was observed in the S+V group. In contrast, the V group showed a 59 % reduction in mean values of number of parasites and the complete absence of parasites in 8/10 animals.



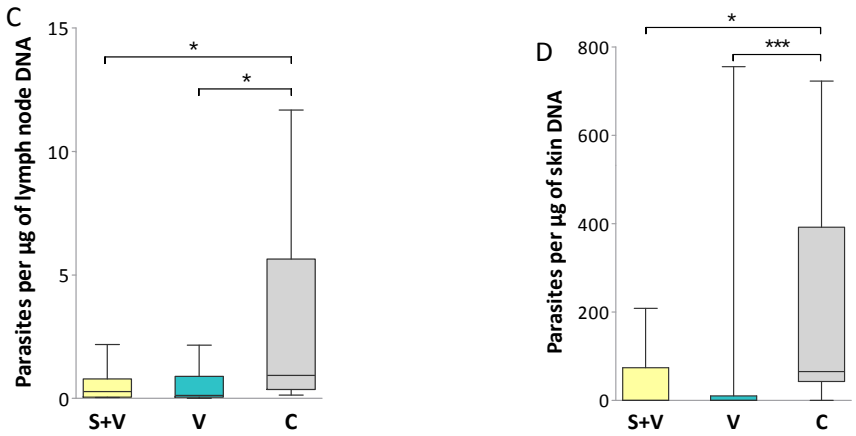


Figure 31: Box plot of qPCR analysis showing the absolute number of parasites detected per µg of spleen (A), liver (B), lymph node (C), and skin (D) comparing the three studied groups: naked DNA-vaccinated and sirolimus-treated (S+V), naked DNA-vaccinated (V) and control (C) group (* $P < 0.05$, *** $P < 0.001$).

Analysis of the immune response:

Arginase activity and IgG production as immune related indicators

All hamsters seroconverted and had IgG antibodies against CTLA at the time of sacrifice at 5 weeks post-infection. *Leishmania*-specific IgG production was significantly reduced in V animals when compared to C ($P = 0.0475$), as was the case between V and S+V ($P = 0.0180$) (Figure 32). In contrast, similar levels of *Leishmania*-specific antibodies were detected in S+V and C groups.

As regards arginase activity, no statistical differences were observed between groups. However, the mean values show a 1.18-fold increase in arginase activity in S+V animals when compared with V and C groups (Figure 32).

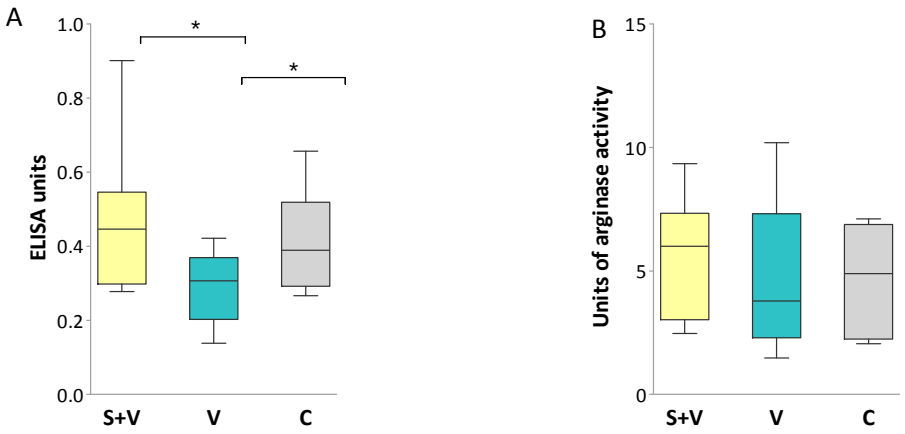


Figure 32: Comparison of IgG levels (A) and arginase activity units (B) of vaccinated hamsters that received (S+V) or not (V) the sirolimus treatment and the control group (C) 5 weeks post-challenge (* $P < 0.05$, *** $P < 0.001$).

Cytokine and immune markers production

Expression of cytokines —IFN-γ, TNF-α and TGF-β — and the Treg immune marker FoxP3 was studied and detected by the day of sacrifice in spleen samples of all experimental groups. Overall, V and S+V groups expressed similar patterns of studied molecules expression, where more expression of TNF-α, TGF-β, and FoxP3, with a concurrent reduction of IFN-γ was detected when compared to non-vaccinated animals (Table 12 and Figure 33).

The vaccine itself induced a significant increase in FoxP3 expression when compared with control animals ($P = 0.024$) (1.476-fold increase over C values), that was potentiated by SIR administration ($P = 0.004$) (1.549-fold increase over C group). In case of IFN-γ, although these differences were non-statistically significant, there is a tendency for both vaccinated groups (V and S+V) in lowering IFN-γ expression (0.578 and 0.602-fold, respectively) compared to the C group. TNF-α production was similar for C and V groups, whereas SIR administration induced a 1.240-fold increase over C values ($P = 0.048$). Lastly, TGF-β expression was also promoted in both vaccinated groups, resulting in a 1.2-fold increased production over control animals that was significant for S+V hamsters (P = 0.048). These results indicate that protection in spleen was achieved even under a reduced expression of IFN-γ and an increase on FoxP3 expressing cells.

Melt curves assessed the specificity of our amplification products.

Table 12: Fold increase of median values over C group (Q1-Q3), and *P*-values for the transcription of target cytokines and immune markers within spleen samples.

TISSUE	GENE	C	V	S+V	V vs C	S+V vs C	S+V vs V
SPLEEN	***IFN-γ***	1	0.578 (0.369-1.239)	0.602 (0.301-0.968)	*P* = 0.304	*P* = 0.214	*P* = 0.696
	TNF-α	1	1.090 (0.933-1.322)	1.240 (1.029-1.338)	*P* = 0.635	***P* = 0.048***	*P* = 0.573
	TGF-β	1	1.230 (1.059-1.650)	1.282 (1.165-1.619)	*P* = 0.106	***P* = 0.048***	*P* = 0.829
	FoxP3	1	1.476 (1.147-1.885)	1.549 (1.324-1.677)	***P* = 0.024***	***P* = 0.004***	*P* = 0.897

Acronyms: C (control group), V (vaccinated group), S+V (sirolimus-treated and vaccinated group), IFN-γ (Interferon γ), TNF-α (Tumor necrosis factor α), TGF-β (Transforming growth factor β), and FoxP3 (fork head box P3) (* $P < 0.05$).

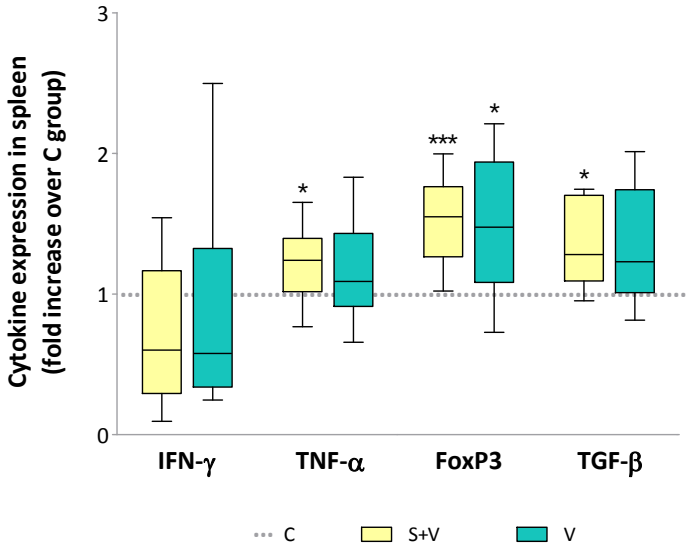


Figure 33: Fold increase of median values of target cytokines and immune markers expression over C group (represented as a dotted gray line) within spleen samples of *L. infantum*-challenged hamsters. Acronyms: C (control group), V (vaccinated group), S+V (sirolimus-treated and vaccinated group), IFN-γ (Interferon γ), TNF-α (Tumor necrosis factor α), TGF-β (Transforming growth factor β), and FoxP3 (fork head box P3) (* $P < 0.05$, *** $P < 0.001$).

DISCUSSION

This work was motivated by the urgent need of safe, cheap, and stable compounds to treat and prevent protozoan tropical diseases that are responsible for an important number of new infections and deaths every year. Different bioenergetic modulators previously used in diagnostic and anticancer studies were hypothesized to be susceptible for drug repurposing in order to manage and prevent malaria, visceral leishmaniases, Chagas disease and human African trypanosomiases. In addition, we tested if a bioenergetic modulator such as sirolimus could enhance a vaccine against a parasitic disease.

- ***In vitro* validation of bioenergetic modulators as therapy for diseases caused by highly glycolytic parasites**

Our results show that bioenergetic modulators have greater efficacy on those parasites that are more dependent on glycolysis, as is the case for *T. brucei* and *P. falciparum* (Michels et al. 2006; Schalkwyk et al. 2008; Salcedo-Sora et al. 2014). We found that a major number of tested compounds were effective against these two parasites, and lower doses were required. Apart from the specific efficacy of the compounds in the inhibition of target enzymes, such differential parasiticidal activity profile of the studied compounds may be due to the contrasting life-condition adaptations and physiological particularities of each organism. Thus, whereas *T. cruzi* and *Plasmodium* spp. are hosted in the glucose-rich cytoplasm of the mammalian host cell, *T. brucei* multiplies in the host bloodstream thereby exploiting its serum glucose levels, and *Leishmania* spp. show special metabolic features as a result of their adaptation to the low glucose concentration in parasitophorous vacuoles (McConville et al. 2007; Naderer and McConville 2008). Despite glucose oxidation playing an important role in *Leishmania* spp. and *T. cruzi*, these parasites are more dependent on amino acid and fatty acid catabolism as a source of carbon and energy at the amastigote stage (Bringaud et al. 2006) and therefore could be less susceptible to glycolysis inhibitors.

DCA is the only bioenergetic modulator we have found to have anti-parasitic activity at the asexual replicative stages within the host of the three genera of parasites studied. It reduces the parasite burden of infected cells in trypanosomatids and the number of infected cells in *L. infantum* in a dose-dependent manner. The main target of DCA is pyruvate dehydrogenase kinase (PDK) (Stacpoole et al. 1998). Genes encoding for PDK have been found in *T. brucei, T. cruzi*, and *L. major* (Parsons et al. 2005). However, as mentioned in the introduction of this work, bloodstream *T. brucei* is known to have an alternative mitochondrial oxidase non-

dependent on pyruvate metabolism, and therefore, PDK is not involved. This fact suggests there must be other possible targets of DCA still unknown in this stage of *T. brucei*. The high IC_{50} observed for DCA in *T. cruzi* may suggest that this is not an essential target at this stage, however low accessibility of the compound to the parasite enzyme and or its specificity may also be a cause for its reduced efficacy. No PDK genetic sequences have been identified in *P. falciparum* (Ward et al. 2004). However, our results show a dose-dependent inhibition of parasite growth by DCA on the intra-erythrocytic stage of *P. falciparum*, suggesting additional effects of this compound on other essential steps for parasite survival, probably not host cell dependent due to the basic metabolism of erythrocytes. The ability of this compound to restore mitochondrial activity may also lead to a decrease of pyruvate availability for other biosynthetic pathways, such as alanine biosynthesis (Tielens 1998; Tielens and van Hellemond 2009). The parasite-killing activity of this drug could, therefore, be enhanced by a lack of key proteins for parasite survival. Whilst several studies have shown the efficacy of DCA in rapidly reducing the concentration of lactic acidosis, a common complication of severe malaria strongly related to death in children (Krishna et al. 1996; Agbenyega et al. 2003), this is the first time that its antiplasmodial activity has been demonstrated.

In addition, DCA activity may also be boosted through actions triggered in the host cell. Many protozoans such as *Leishmania* and *Toxoplasma* activate and stabilize HIF1A in the host cell (Wiley et al. 2010; Singh et al. 2012). HIF1A controls the cellular response to low oxygen stress inducing the transcription of genes related to the metabolic switch from oxidative phosphorylation to glycolytic profile, angiogenesis, and apoptosis inhibition (Semenza 2012). Such a strategy may help these parasites to survive by taking advantage of the increase in macromolecules provided by glycolytic metabolism and the induced inhibition of host cell apoptosis. It has been recently described that the DCA anti-tumor effect also takes place by decreasing HIF1A activity in cancer cells (Kumar et al. 2012; Sutendra et al. 2013). As a consequence, the observed DCA parasite burden reduction may be related to a decreased HIF1A activity and, in turn, the loss of the metabolic advantages that the host cell provides to its undesirable guests.

We found that the glycolysis inhibitor 3BP showed anti-parasitic activity on *T. brucei*, *T. cruzi* and *L. infantum* intracellular amastigotes, with potential for VL and American and African trypanosomiasis treatment. Previous studies have reported some activity on *T. brucei*, inhibiting motility and growth (Barnard et al. 1993), which corroborates our results.

Nevertheless, we found no efficacy of 3BP against *P. falciparum* asexual replicative forms despite these parasites being more dependent on glycolysis than the intracellular amastigote stage of *Leishmania*. The main target of 3BP is GAPDH, an enzyme of the glycolytic pathway localized mainly in the glycosomes of kinetoplastida. Some *Leishmania* species also have a different cytosolic GAPDH isoenzyme, shown to play a significant role in VL (Zhang et al. 2013). The efficacy we observed on *Leishmania* may therefore be due to the inhibition of both isoenzymes. A predictive model led to the conclusion that slight depletions of glycosomal GAPDH result in complete impairment of growth and ATP synthesis in *L. infantum* amastigotes (Subramanian et al. 2015), thus reinforcing the role of this enzyme for parasite-killing purposes. Furthermore, an increase in GAPDH expression in *Leishmania* spp. has been related to a resistance to NO (Rios et al. 2015), a basic molecule for intracellular parasite clearance (Mauël et al. 1991). Blocking GAPDH with 3BP would avoid this resistance and allow parasite-killing. According to some authors, 3BP could not be a therapeutic option because of its toxicity (Barnard et al. 1993), but nevertheless it was apparently safe in *in vivo* trials performed in rodents (Ko et al. 2004; Schaefer et al. 2012).

LND and 2DG are compounds in which the mechanism of action is based on the interference with hexokinase activity (Nirenberg and Hogg 1958; Paggi et al. 1988). LND has been reported to also inhibit that enzyme of *T. brucei* (Chambers et al. 2008). The efficacy of LND on trypanosomatids has been described and tested on several species, including *T. cruzi* epimastigotes (Turrens 1986), *T. brucei* bloodstream forms (Chambers et al. 2008), and *L. mexicana* promastigotes (Turrens and Cazzulo 1987). In the case of trypanosomes, we found an IC_{50} for *T. cruzi* amastigotes very close to that previously observed in epimastigotes (Turrens 1986), and the observed effect of LND on the bloodstream stage of *T. brucei* confirmed the results previously obtained by Chambers et al. (Chambers et al. 2008). In contrast, no effect was detected on either *L. infantum* promastigote or amastigote stages. Surprisingly, despite the *T. brucei* bloodstream form relying entirely on glucose catabolism through the glycolytic route for ATP generation, 2DG did not interfere with its growth in our *in vitro* model. In contrast, 2DG reduced the intracellular burden of *T. cruzi* amastigotes, although high doses were required, which reflects the fact that this parasite stage also has alternative sources of carbon to feed the pathway. In addition, this result may suggest the existence of an alternative hexose transporter in this parasite stage, as the facilitated transporter in charge of glucose uptake seems to be absent in *T. cruzi* amastigotes (Silber et al. 2009). On the contrary, hexose uptake, despite its low level within parasitophorous vacuoles, is known to be essential for

parasite survival and growth in the case of *L. infantum* amastigotes (Burchmore et al. 2003; Naderer and McConville 2008). However, 2DG showed no effect on *Leishmania* viability, probably due to the limited access of this compound to the closest environment to the parasite in the phagolysosome. Both 2DG and LND showed antiplasmodial activity on *P. falciparum*. 2DG has been previously reported to interfere with glucose uptake in *P. falciparum*, with parasite glycolysis, and with the synthesis of glycosylated macromolecules that play a key role in the intra-erythrocytic stage of development of trophozoites, thus reducing their viability (Naik et al. 2000; Santos de Macedo et al. 2001; Schalkwyk et al. 2008). This is the first time LND has been used as an antiplasmodial compound. Because it resulted in a 50 % reduction of *P. falciparum* viability at concentrations of 209 µM, it emerges as a potential candidate for future malaria treatment.

In this study, we have confirmed the previously described MET antiplasmodial activity (Sweeney et al. 2003) and report, for the first time, its efficacy against *T. cruzi* and *T. brucei*. MET is thought to block the mitochondrial respiratory-chain complex I. The presence of mitochondrial complex I in Trypanosomatida has been predicted, and all of its components identified, but to this date, no evidence has been found of its involvement in *T.brucei* energy metabolism (Opperdoes and Michels 2008). In the case of *P. falciparum*, there is an alternative NADH dehydrogenase in substitution of the classic complex I (Biagini et al. 2006). Complex I inhibition by MET leads to metabolic and energetic stress, and activation of AMPK (Fryer et al. 2002), thus causing down regulation of energy-consuming processes and pushing the cell into a quiescent state (Fryer et al. 2002; Viollet et al. 2012). However, if cells lack the ability to cope with energetic stress, as may be the case for tumor cells and glycolytic parasites, a metabolic crisis develops leading to death. In addition, the inhibition of the mitochondrial respiratory-chain complex I by MET in host cells reduces O_2 consumption as the final acceptor of electrons in the electron transport chain. This would lead to an increase in intracellular O_2 which promotes hydroxylation of HIF1A by procollagen-proline dioxygenase (P4HA) and its destruction in the proteasome (Takiyama et al. 2011; Takiyama and Haneda 2014). As described above, HIF1A appears to be a key component for parasite survival, perhaps opening new avenues for anti-parasite therapeutic intervention. Antiplasmodial activity could also be explained by the disruption caused by MET on metabolic pathways related to folates, responsible for purine and pyrimidine synthesis, for catabolism of several amino acids, and for glutathione synthesis, thus hindering parasite survival (Faja et al. 2012; Bridges et al. 2014).

The mTOR route is a highly conserved pathway in eukaryotic organisms (van Dam et al. 2011), regulating the balance between catabolism and anabolism according to cellular needs (Sabatini. Laplante. 2013). It acts on energy-generating routes, DNA transcription, synthesis of biological molecules, autophagy, and cell death (Sabatini. Laplante. 2013; Shimobayashi and Hall 2014). In trypanosomatids, the TORC2 ortholog is the only TOR complex susceptible to SIR inhibition. TORC2 is involved in cytoskeletal actin reorganization, playing a key role during host cell invasion and parasite survival (Barquilla et al. 2008). In our model, SIR did not affect the viability of intracellular *T. cruzi* and *L. infantum*. In fact, a slight increase in parasite load was observed. Similar results have been reported in other intravesicular parasites, such as different *Leishmania* species (Schaible et al. 1999; Pinheiro et al. 2009; Jaramillo et al. 2011) or even in *P. berghei* during its intra-hepatic stage when the parasite inhabits non-acidified parasitophorous vacuoles (Lingelbach and Joiner 1998; Hanson et al. 2013). It has been suggested that mTOR inhibition by SIR triggers autophagy and other processes in the host cell, providing them with nutrients for their survival and parasite replication (Pinheiro et al. 2009; Cyrino et al. 2012; Brunton et al. 2013). Such processes in the host cell may provide a feasible explanation, since autophagy has been reported to promote the generation and maturation of replicative vacuoles of other intracellular pathogens, such as *Coxiella* sp. (Gutierrez et al. 2005). Moreover, the mTOR route is related to the production of NO (Weichhart et al. 2008) thus impairing intracellular parasite clearance by blocking mTOR activity when SIR is administered.

In case of the intraerythrocytic stages of *P. falciparum*, our results concur with previous studies (Angus Bell et al. 1994; Yap et al. 2014). Erythrocytes have high avidity for SIR, therefore most of it reaching the bloodstream is sequestered in RBCs and bound to their immunophilin binding protein FKBP12 (Trepanier et al. 1998). RBCs have an iron dependent mTOR pathway that regulates hemoglobin production and erythrogenesis. However, SIR has shown only modest effect on RBCs in contrast to other mTOR inhibitors responsible for anemia and the decrease in different blood cell populations (Knight et al. 2014). Besides, whilst some authors have even indicated the expression of mTORC1 and C2 proteins in the proteome of mature erythrocytes, there is no evidence of functional proteins (Alessandro et al. 2010; Hanson et al. 2013). It may be assumed, therefore, that erythrocytes concentrate SIR, exposing the intracellular *P. falciparum* forms to high concentrations, and that the observed growth inhibitory effect relies purely on the parasite-drug interaction. To date no *Plasmodium* orthologs of mTOR have been found (Brennand et al. 2011; van Dam et al. 2011). Nevertheless,

SIR has been reported as developing its action by inhibiting the activity of the only FKBP described in *P. falciparum* (PfFKBP35), which is involved in protein folding and stabilizing, and is an essential chaperone, thus affecting parasite viability (A Bell et al. 1994; Kumar et al. 2005; Monaghan and Bell 2005).

In summary, the activity of several antineoplastic drugs was studied in different intracellular parasites due to their metabolic resemblances to cancer cells. However, the mechanisms of action are still undiscovered, as metabolism of these parasites is only partially demonstrated. The results show that metabolic pathways related to energy and biomass production are promising targets. This broad principle might offer an economic way to prevent a wide range of neglected parasitic diseases by simply repurposing existing commercially-approved drugs to this new indication.

- ***In vivo* validation of bioenergetic modulators as therapy for diseases caused by highly glycolytic parasites**

The next step in validating the suitability of a molecular target and its related inhibitor drugs is to perform *in vivo* studies. In the second chapter of this work different bioenergetic modulators that revealed effectiveness in the *in vitro* study on reducing the number of parasites in stages responsible for causing disease in the host were studied on rodent models of their related disease.

Unfortunately, our results showed that the assayed compounds neither reduced parasite burden nor expanded the life span of infected animals in any of the analyzed *in vivo* models. Living organisms are much more complex and, in most cases, it is not possible to extrapolate the *in vitro* obtained data to the *in vivo* system. Even ignoring the variability that arises from the dose of drug reaching tissues due to the pharmacokinetic behavior and metabolism of the compound, the reactions triggered in different cell populations that constitute the whole organism modulate the final overcome of the treatment. We describe, hereunder, possible explanations to the otherwise interesting findings we recorded in our *in vivo* experiments.

· Neither DCA nor 2DG contributed to murine malaria management

In the second chapter of this work, two different concentrations of DCA and 2DG were tested in a murine model of *P. yoelii* malaria. As described in the previous chapter, the treatment of cultured *P. falciparum*-infected RBCs with 2DG or DCA showed similar antiplasmodial values to those provided by chloroquine (91.3 % and 89 %, respectively, versus 89.9 % CQ) turning these drugs into suitable candidates for the *in vivo* experiment. In addition, several studies showed the efficacy of DCA in quickly reducing lactic acidosis, a common complication strongly related to death in severe malaria mainly affecting children (Krishna et al. 1996; Agbenyega et al. 2003). Furthermore, DCA has been shown to increase the life span of rats infected with *P. berghei* (Holloway 1995). DCA was thereby selected to study whether its ability to keep patients alive longer may not only be the result of controlling lactic acid levels in blood, but also due to the antiplasmodial activity, as our *in vitro* data suggests. For that purpose, two different doses were assayed, including 100 mg/kg used by Holloway (Holloway 1995) and a higher dose. Our negative results on reducing parasite loads suggest that the increased life span observed in malaria-diseased rats treated with DCA is not a direct consequence of the effect of this drug on controlling parasite dissemination, but is mostly due to the ability of this compound to buffer acidosis levels.

2DG seemed to be also a reasonable therapeutic treatment for controlling parasitemia that might lead to curative efficacy, as shown in the previous *in vitro* test. However, the *in vivo* analysis of 2DG at assayed doses and for the model of murine malaria was ineffective: 2DG did not control the progression of the parasite and, moreover, no higher doses of this compound resulted in a better outcome of the disease. Furthermore, adverse events were recorded as described below. Two out of five animals treated with 2 g/kg 2DG showed severe trembling on day 7 post-treatment. These doses are described in the bibliography (Tagg et al. 2008), and no adverse effect was described, even when drugs were administered for longer periods (more than 20 days). Conversely, other authors referred to anorexia, weight decrease, and death in mice at doses higher than 250 mg/kg (Marsh et al. 2008). The rest of the observed symptoms can presumably be due to the advance of murine malaria. Hemoglobinuria detected in 3 mice in the high-dose 2DG group may be derived from the fact that 2DG and DCA might be interfering with the sole mechanism mature erythrocytes have to regenerate NADPH, the Pentose Phosphate Pathway (PPP). It is known that 2DG-derived metabolites cannot be that efficiently used by the PPP (Ferraris et al. 1981) (Chi et al. 1987), and DCA has already been

demonstrated to block that route (Preter et al. 2015), thereby making RBCs more sensitive to oxidative stress, thus inducing their early lysis, which can be potentiated by plasmodial infections. A genetic study performed in Gambia indicated that G6PDH deficiency —for the main result is a reduced functioning or complete abrogation of the PPP route— confers protection from cerebral malaria but increases the risk of suffering from severe malaria-associated anemia as it contributes to RBCs rupture, which supports this hypothesis (Shah et al. 2016).

A feasible reason to explain why the *in vitro* experiments were successful, but no parasiticidal effect was detected in the *in vivo* approach, could be related to the model used. The *P. yoelii* murine model is widely accepted for the *in vivo* study of experimental malaria, but there are many differences between models that should be considered. Although *P. yoelii* also invades mature erythrocytes as *P. falciparum* does, it preferably parasitizes reticulocytes, which have a more intense metabolism than mature RBCs, and thereby can end in an alteration of the drug activity. In addition, *P. yoelii* has an 18 h asynchronic cycle within the host cell (Gautret et al. 1994), while *P. falciparum* has a synchronic cycle that lasts 48 h (Bagnaresi et al. 2009). This means that *P. yoelii* multiplies 2.6 times faster than *P. falciparum*, which can be translated into a higher number of parasites in early moments of infection when drugs have not yet reached therapeutic levels. As a future prospect, these compounds should be tested in a *P. yoelii in vitro* model to compare them with the results provided by the *P. falciparum* model, to rule out strain resistances.

Malaria is a severe disease that kills millions every year. In spite of its importance, the evolution of the pathological process is still controversial. Some authors hold that plasmodial infection derived symptoms, apart from the problems directly caused by parasite replication, are of an immune nature, with the cytokine storm concept being central for explaining the pathology (Clark et al. 2008). However, these pro-inflammatory mediators responsible for causing disease are also basic tools of the cell mediated immunity (CMI) that exerts its inhibitory pressure on parasite growth (Reviewed by Angulo and Fresno (Angulo & Fresno, 2002)). For example, the administration of anti-TNF-α therapies to *Plasmodium*-infected children was not only associated with reduced fever (van Hensbroek et al. 1996), but also with a parasite clearance delay when combined with other parasiticidal compounds (Looareesuwan et al. 1999). As 2DG has been previously demonstrated *in vitro* to reduce TNF-α production levels in human monocytes in a dose dependent manner (Dietl et al. 2010), a better outcome

of the disease was expected by administering glycolysis inhibitors in our study. However, no apparent health improvement was detected in DCA and 2DG treated mice. Having said that, one hypothesis is that the ability of glycolysis inhibitors to reduce TNF-α production might be another cause for therapy failure in our study, as TNF-α levels post-treatment may be low enough to allow parasite growth. As a limitation of our work, no TNF-α production was studied to evaluate this possibility.

· DCA, 3BP and MET did not alter parasite numbers when administered to Chagas disease and HAT models.

In chapter 2, DCA, 3BP, and MET were tested in a murine model of HAT and Chagas disease. Although previous *in vitro* experiments performed in the first chapter of this work led us to hypothesize these compounds could be good anti-trypanosomatids, no effect was recorded in reducing parasite burdens and increasing life span of infected animals: DCA and 3BP-treated mice showed similar parasite burdens to animals administered with the vehicle.

Surprisingly, mice treated with MET died within 48 h post-administration. In a recent report, Brima et al. combined the effect of a high fat diet and the administration of MET (50 mg/kg/d) to CD-1 mice in order to study the protective effect of the metabolic syndrome against *T. cruzi* infections. In their work, MET drastically reduced mortality of infected mice, and it was also responsible for lowering the parasite burden (Brima et al. 2015). As their *in vitro* approach did not show parasiticidal effect on *T. cruzi*-infected human foreskin fibroblasts after 72 h of co-culture with MET, the authors suggested the observed effect in the *in vivo* study may be due to the immunomodulatory activity of MET. MET activates AMPK in macrophages that leads to a reduction in the production of pro-inflammatory cytokines —their overproduction leads to pathological processes in the organism, which are frequently considered typical symptoms of the disease— although not enough to constrain the control of the parasite. These results are not in accordance with the ones obtained in our murine model. A feasible reason to explain the sudden death experienced by our mice, and therefore the difference in success with the experiment performed by Brima et al, may be due to the doses employed. Previous studies used higher concentrations of MET administered for longer periods to the same mouse phenotype as the one used by us, and no adverse events were described related to the administration of this compound (Novakovic et al. 2013). However, mice used in these works were not infected with trypanosomatids. The known hypoglycemic effect of MET, in

conjunction with the general reduction of blood glucose during Chagas disease and sleeping sickness (Wang et al. 2008; Nagajyothi et al. 2013), may have caused hypoglycemia levels that were fatal.

Although *T. brucei* is highly dependent on glucose consumption (Michels et al. 2006; Chambers et al. 2008) and no host cell is needed to complete its biological cycle in the vertebrate host, the administration of glycolysis modulators did not affect parasite multiplication in infected-mice. Also, trypanosomatids have been described to depend on the PPP (Maugeri, Cannata, and J.-J. Cazzulo 2011) and at least DCA is known to abrogate this route (Preter et al. 2015), in addition to other possible effects described for each of the tested compounds explained in the first chapter of this thesis. No reduced parasite burdens were detected after the administration of these compounds. A possibility that may help us to explain the failure of this strategy on the *in vivo* model, while *in vitro* results were promising, and that could also be applied for all the tested models in this thesis, is related to the possibility of not reaching therapeutic levels in the closest environment to the parasite, as *T. cruzi* resides within host cells, and *T. brucei* is also distributed in lymphatic vessels and adipose tissue (Trindade et al. 2016), and can also reach the cerebrospinal fluid.

· The *in vivo* assay of DCA and 3BP in the hamster model of VL showed no reduction of parasite burdens and suggests a possible involvement of immune inactivation.

In chapter 2, DCA and 3BP were assayed on *L. infantum*-infected hamsters to check their anti-leishmanial effect observed in the *in vitro* assay. Unfortunately, no reduction of parasite burdens in target organs was detected for treated-animals when compared to the control group. This finding could be mirroring the difficulty of these compounds to reach the parasite environment, as *Leishmania* amastigotes reside within parasitophorous vacuoles. Although assayed concentrations in this experiment demonstrated the tumor killing properties as described in literature, thus indicating that doses in this study are able to reach therapeutic concentrations in tissues, no effect was recorded in parasite viability in our *in vivo* approach, thus lower concentrations within parasitophorous vacuoles may be responsible for the reduced effect.

In our VL model, we were able to analyze immune parameters which helped us to try to gain an insight in the immune response developed by infected and treated animals. Overall, pro-

inflammatory cytokines have been recurrently described to be produced during VL progressive disease in hamsters, together with anti-inflammatory cytokines that are related to an impaired control of the disease (Melby, Chandrasekar, et al. 2001; Garg and Dube 2006; Loría-Cervera and Andrade-Narváez 2014). In our study, both pro- and counter-inflammatory cytokines were detected by the day of sacrifice in spleen and liver samples of infected hamsters that received no treatment, confirming the previous statement. Surprisingly, reduced levels of both types of immune response were recorded for animals that received DCA or 3BP with the same parasite load as untreated hamsters.

Taken as a whole, the inhibition of glycolysis in immune cells seems to be a feasible reason to explain the recorded events in our *in vivo* model of VL, where reduced levels of pro- or anti-inflammatory cytokines, as well as other immune-related parameters, such as immunoglobulin levels or arginase activity, were detected even in the presence of high parasite burdens in target tissues of animals treated with bioenergetics modulators. At first, aerobic glycolysis was described as an energy obtaining system for cancer cells, which was also generalized to all proliferating cells. Cells committed to proliferation generally switch their metabolism to aerobic glycolysis for ATP production while producing biomass to support replication and ensuring protection from free radicals (reviewed by (Vander Heiden et al. 2009)). Following this line, activation of immune cells implies higher energetic demands that must be fulfilled through metabolic reprogramming, switching from a quiescent state towards a Warburg metabolism-committed activated state. The immune system is a critical ally to combat pathogenic insults, as well as to maintain tissue homeostasis in vertebrate organisms. In response to surrounding stimuli, innate and adaptive immune cells leave their quiescent state by experiencing a respiratory burst —that mediates their activation— followed by a metabolic reprogramming to face important changes destined to overcome the aggression. These metabolic changes dictate the fate of both innate and adaptive immune cells; whereas M2 and regulatory T cells (Tregs) are fatty acid oxidation and mitochondrial respiration-committed, M1 and effector T cells (Th1/2/17) metabolism rely more on aerobic glycolysis (Michalek et al. 2011; Ganeshan and Chawla 2014; O'Neill, Luke A.J.; Kishton, Rigel J.and Rathmell 2016). For this reason, glycolysis inhibition has an impact on the effector function and fate of immune cells differentiation, as has been repeatedly demonstrated by the effect brought about by 2DG in immune cells (Dietl et al. 2010; Shi et al. 2011; Ganeshan and Chawla 2014).

Furthermore, inhibition of glycolysis in activated innate immune cells, such as M1 macrophages, impairs ROS production and their pathogen clearing ability (Peyssonnaux et al. 2007). It must not be forgotten that macrophages are the main host cell of *Leishmania* and can also harbor *T. cruzi* amastigotes. Inhibition of glycolysis may then directly affect not only the parasite, but also decide the fate of macrophage polarization and then abrogating their ability to destroy undesirable guests. In the case of T lymphocytes, glycolytic metabolism relies on the differentiation towards CD4+ Th1, Th2, and Th17 effector cells, while Treg and memory T cells rely more on fatty acid oxidation and OXPHOS (Michalek et al. 2011; Buck et al. 2015).

In our hamster model of VL, the lower levels of cytokine expression in animals receiving 3BP or DCA compared to animals that remained untreated may be explained following the immune-related hypothesis previously posed. A general reduced expression pattern of cytokines can be noted between animals that received the assayed glycolysis inhibitors and animals that remained untreated. In the case of TGF-β, levels of this cytokine was significantly lower in the liver of animals treated with 3BP, but were not affected in spleen samples by either of the tested compounds. TGF-β is a cytokine produced by Tregs, neutrophils, and alternatively activated macrophages which is associated with susceptibility and immunosuppression, and its levels are known to rise during VL in the hamster model (Fazzani et al. 2011). The proper functioning of Treg does not rely as much on glycolysis like other T activated cells, but it is known that the inhibition (or deletion) of mTOR promotes Treg differentiation and activity (Turnquist et al. 2007; Delgoffe et al. 2010). Therefore, it would have been interesting to further analyze the effect of 3BP and DCA on Treg cells.

A metabolic switch towards a glycolytic profile has also been described when B cells are activated. Although this glycolytic reprogramming is more balanced, and not as dramatic as that described for T cells, it has been demonstrated to play a crucial role in antibody production, as the administration of glycolytic inhibitors resulted in a steep reduction in antibody production (Caro-maldonado et al. 2014). In this referred study, Caro-Maldonado et al. assayed DCA *in vitro* and *in vivo* and confirmed this reduction in antibody production and B cell proliferation, although the general numbers of both B and T cells in peripheral blood were not altered. No reduction in IgG levels were detected for DCA-treated mice compared to untreated animals, but the results provided by 3BP-treated animals follow the line set by these authors. This difference observed between our two experimental treated groups may be explained as DCA is not as potent a glycolytic inhibitor as 3BP, since DCA slows down the

glycolytic flux by activating the mitochondrial metabolism. This reduced glycolytic flux may be enough to maintain antibodies production.

In our study, significant differences in arginase activity levels were detected between groups, and may be explained due to the natural ability of *Leishmania* to induce macrophage STAT 6 activation and STAT 6-dependent arginase expression, which is not required, but is amplified by Th2 cytokines (Osorio et al. 2012). The arginase results in our study can be explained following this reasoning: infected animals that were treated with 3BP and DCA showed lower activity of this enzyme in spleen compared to the control group —although higher parasite burdens were recorded for this organ— because of the lower levels of Th2 cytokines when compared to untreated animals. Arginase is a known enzyme related to the M2 macrophage profile and it is a direct competitor of iNOS for L-arginine (reviewed by Rath et al. (Rath et al. 2014)), thus it would have been interesting to evaluate NO production or iNOS expression, related to the M1 phenotype, in order to have a more complete view of the effect of glycolysis inhibitors on the total macrophage population and give support to our findings. However, the natural low induction of iNOS activity mediated by IFN-γ in hamsters (Perez et al. 2006) would have made its analysis into a difficult, and possibly meaningless, task.

The histological findings recorded in this study —like the reduced number of granulomas detected in the liver of hamsters treated with DCA or 3BP, and the lower peri-portal mononuclear infiltrate— could also be explained, as 3BP-treated animals showed lower inflammatory signals in liver than animals that received DCA, and these concurrently presented with lower inflammation signs than untreated animals. It is known that granuloma maturation is a cytokine-dependent process in which TNF-α plays a major role (Kaye et al. 2004; Vasco Rodrigues et al. 2016). The lower level of TNF-α detected in animals treated with 3BP compared to DCA-treated animals, and both with untreated controls, explains the lower number of mature hepatic granulomas observed in this study.

All the studied diseases share an important characteristic that must be kept in mind, and may constitute another reason to explain the inefficacy of glycolysis modulators on *in vivo* tests: malaria, HAT, Chagas disease and VL have been frequently described as diseases with an inflammatory profile where a balance between pro- and counter-inflammatory cytokines is involved in protection and immunopathology (MacLean et al. 2004; Kennedy 2006; Stanley and Engwerda 2007b; Clark et al. 2008). For that reason, it is not rare to find exacerbated

infections in which the resolution depends on CMI-clearance during anti-TNF-α therapies used for improving the symptomatology of concurring inflammatory diseases —such as rheumatoid arthritis or psoriasis— as pro-inflammatory cytokines are needed for controlling parasite growth (Tumang et al. 1994; Geraghty et al. 2007; De Leonardis et al. 2009; Ali et al. 2013). The ability of glycolysis inhibitors to block the activation of immune quiescent cells and to turn into activated cells unable to maintain their cytokine production, may, therefore, be a feasible reason to explain the non-reduction of parasite burdens detected in our rodent models.

Furthermore, the general inflammatory profile that accompanies these parasitic diseases modulates many reactions within the host cell that leads to NO and ROS production, known molecules that impair the correct functioning of the mitochondrion (Everts et al. 2012). This concept is of special interest in the case of DCA, where the main mechanism of action derives from the recovery of the loss of mitochondrial activity in many cancer cell types. Thus, when administered to an organism with an already triggered inflammatory response —as happens in individuals infected with trypanosomatids or *Plasmodium* parasites—, DCA activity on mitochondrion may be useless, as the mitochondrion is no longer available, as suggested by Clark et al. (Clark et al. 2008).

The lack of parasiticidal effect observed in the *in vivo* approach when these compounds efficiently controlled parasite growth in the *in vitro* assay, suggests that activated immune cells are more sensitive to glycolysis inhibition than the intracellular form of these parasites. Thus, higher concentrations of these compounds in the closest environment to the parasite are needed to efficiently control their growth. Specifically addressed glycolysis inhibitors may be a reasonable option to efficiently target infected cells, avoiding undesirable effects on immune metabolism and adverse events in the host. Taking these results together, we can conclude that non-specifically addressed glycolysis inhibitors may not be an appropriate choice for treating diseases caused by highly glycolytic parasites.

- **The mechanistic Target Of Rapamycin as a therapeutic target to enhance the response of a vaccine against VL**

The search for a prophylactic solution to constrain the spreading of VL is already a challenge. Different vaccination approaches have been assayed up until now, with variable results, but the secret of how to achieve the perfect combination of a balanced safe and protective

immunization still remains locked. This is the first time that the immunosuppressive drug SIR is studied as a possible modulator of the immune response against *Leishmania* during an immunization protocol. For that purpose, three different experimental groups were defined (vaccinated, vaccinated+SIR-treated, and control) and the effect on parasite burdens and immune mediators were analyzed after a posterior *L. infantum*-challenge.

As the obtained results reveal, the naked DNA vaccine containing *KMPII, PAPLE22, TRYP* and *LACK* sequences designed in our laboratory induced partial protection to an *L. infantum* challenge in our hamster model by reducing the burden of parasites in skin and lymph node target organs. The administration of SIR during the expansion and contraction phase of the immune response triggered by the vaccine enhanced the protective power provided by the vaccine itself, the observed protection expanding to spleen.

In previous studies, we have already shown that multigenic DNA-vaccines containing the same genetic sequences of *Leishmania* —KMPII, LACK, PAPLE22 and TRYP—achieved partial parasitological protection of *L. infantum*-infected hamsters (Todolí et al. 2012). However, when a similar DNA vaccine containing the TRYP, LACK, KMPII and GP63 gene sequences was assayed in the canine model of VL, it resulted in an impaired control of the disease, although a slowed-down organ colonization was observed (Rodríguez-Cortés et al. 2007). Other studies have shown the variable protective power of these genes when individually injected. A *KMPII* DNA-vaccine induced protection against *L. donovani* infection in hamsters by inducing a mixed Th1/Th2 immune response with up regulation of Th1 cytokines production —IFN-γ, TNF-α and IL-12— and decreased levels of IL-10 (Basu et al. 2005), and similar protection was also achieved against an *L. chagasi* challenge (da Silva et al. 2011). The administration of the *PAPLE22* DNA sequence also resulted in a reduction of circulating parasites and humoral response in hamsters (Fragaki et al. 2001). A *TRYP* DNA vaccine was non-protective in a murine model against *L. major* and needed a primer-boost strategy to induce protection (Stober et al. 2007). *LACK* DNA vaccines promoted a strong Th1 response without inducing protection in the mouse model of VL and CL (Gurunathan et al. 1997; Melby, Yang, et al. 2001; Marques-Da-Silva et al. 2005), and vaccine failure was associated with IL-10 exacerbated production (Marques-Da-Silva et al. 2005). However, an intranasal approach provided protection against *L. amazonensis* and reduced parasite load in hamsters and mice infected with *L. chagasi* (Gomes et al. 2007; De Oliveira Gomes et al. 2011). These results reflect the

variable immunogenicity of these genetic sequences, and reveal the need for boosting strategies to potentiate their protective efficiency.

Plasmid DNA vaccines are known to promote antibody production and stimulate CD4+ and CD8+ T cell responses (Gurunathan, Wu, et al. 2000; Huygen 2005). However, serology levels in our study revealed a significant decreased quantity of anti-*Leishmania* specific IgG antibodies in hamsters that received the vaccine alone when compared to the sham group. These reduced antibody levels were already observed in a previous experimental trial of this vaccine and may be explained by the ability of the vaccine to control the aberrant B cell differentiation widely associated with disease progression (Todolí et al. 2012). Surprisingly, the administration of SIR to the immunization protocol was translated into a significant increase in *Leishmania*-specific IgG levels compared to animals vaccinated only, showing similar antibody levels to the control group. Although an association between increased antibody responses due to polyclonal B activation, elevated parasite burden, and the absence of proliferative responses have been described in the VL hamster model (Riça-Capela et al. 2003; Dea-Ayuela et al. 2007), the observed increase in *Leishmania*-specific IgG levels in animals treated with SIR in our study is not accompanied by an increased parasite burden. The effects of SIR on antibody production during an immunization assay with influenza virus have been previously studied (Keating et al. 2013). These authors describe that SIR administration in mice does not alter specific IgG antibodies level compared to infected controls, which is corroborated by our results. Their study brought to light that SIR does not prevent specific antibody formation, but impairs B cell class-switching and germinal center formation, in addition to decreasing the number of T follicular helper cells (Tfh) (Keating et al. 2013). Tfh cells are a CD4+ T subset that coordinate germinal center formation and the production of high affinity antibodies (Crotty 2011). Thus, SIR increases the titer of low affinity immunoglobulins and alters the antibody repertoire specificity that may mediate in protection (Keating et al. 2013). In the case of VL, an increased Tfh infiltration in spleen has been reported during first weeks of infection, leading to B cell maturation and increased IgG production (Rodrigues et al. 2014). In contrast, low avidity antibodies are predominant in asymptomatic, low parasitized, and cured human patients (Tiburcio et al. 2013), as well as in dogs (Neto et al. 2010). For that reason, we suggest that the lack of GC maturation or Tfh infiltration in spleen in early stages of infection may be favorable for controlling VL. Our results suggest that the increased parasite-specific IgG detected in the S+V group may be mediated by an increased low affinity antibodies production induced by SIR

activity, which may be responsible for the reduced parasite burden detected in the spleen in S+V animals.

Arginase activity is considered as another disease progression indicator, as it is a metabolic marker of alternatively activated macrophages related to leishmaniases management failure (Osorio et al. 2012; Abebe et al. 2013). In our study, a tendency for increasing arginase activity levels was detected for SIR-treated group. However, no correlation was found between this enzyme activity in spleen and parasite burden values for the same tissue. Despite both serology and arginase activity having been associated with disease progression and suppression of the protective immune response against *Leishmania*, the increase in both parameters in the SIR-treated group does not correlate with the subsequent increase in parasite burden, suggesting this effect may be produced by the modulation induced by SIR and not due to a defective response against the parasite.

As referred elsewhere in this work, the administration of SIR during the expansion phase of an immune response development is known to increase the number of precursor memory cells, and when administered during the contraction phase, it accelerates the T cell differentiation program towards memory cells (Araki et al. 2009), promoting, therefore, potentiated specific immune responses to a stimulus. Several authors have already demonstrated the ability of this compound to boost different immunization trials against tumors (Li et al. 2012; Diken et al. 2013), viruses (Turner, Shaffer, et al. 2011; Keating et al. 2013), and even intracellular mycobacteria (Jagannath and Bakhru 2012). The protection achieved in these models was mediated by the generation of memory effector CD8+ T and the concurrent increase in IFN-γ production, typical of these cells phenotype. Nevertheless, the aforementioned studies were conducted by analyzing isolated cell populations from *in vivo* tests and directly labelling CD8+ IFN-γ-producing cells, which in our case was not possible due to the lack of reagents available for hamster sample analysis. For that reason, we analyzed the production of some relevant cytokines involved in VL control and pathogenesis in the whole spleen tissue, in order to evaluate the immune response triggered in that organ. Surprisingly, in our study, despite achieving the parasitological protection in spleen experienced by SIR-treated hamsters, the evaluation of cytokines expression in the same tissue revealed a tendency for lowering IFN-γ production, pointing out that the mediated protection in our model may not be conferred by the expected SIR effect, or at least we could not detect it by analyzing of the whole spleen.

Although IFN-γ is a cytokine widely accepted to be involved in *Leishmania* control, it is also known to steadily increase during early stages of infection without a clear contribution to parasite control (Melby, Chandrasekar, et al. 2001; Perez et al. 2006). Recent studies also show that IFN-γ has a paradoxical effect in promoting parasite growth (Kong et al. 2017). Consistently, in our study, vaccinated animals presented lower IFN-γ expression levels in spleen samples than control hamsters. However, only the SIR-treated group showed parasitological protection in this tissue. Our results suggest that our DNA vaccine regulated IFN-γ production by avoiding its over-expression, and therefore its counter-productive effect, which resulted in protection due to additive effects of SIR. Surprisingly, parasitological protection in spleen occurred even under higher expression levels of FoxP3, which may suggest the need to rethink the role of Treg FoxP3+ cells in *L. infantum* infection. Although regulatory T cells have been widely associated with disease progression, it has been described in literature that there are other cell populations that play a more important role in human VL active disease than FoxP3+ Treg cells (Nylén et al. 2007). Furthermore, FoxP3 expression in skin has also been correlated with asymptomatic disease in naturally infected dogs (Menezes-Souza et al. 2011), as well as being inversely correlated in LN with parasite burden in *L. infantum*-experimentally infected dogs (Hosein et al. 2015), and lower levels of this immune marker were detected in spleen and jejunum of naturally infected dogs compared to control animals (Figueiredo et al. 2014; Silva et al. 2014). In addition, other studies show the protective role of Treg expansion in *L. panamensis* and *L. amazonensis* infection, favoring an improvement in the disease (Ji et al. 2005; Ehrlich et al. 2014), probably by relieving the hyper-inflammatory state by IFN-γ downregulation, as may also be the case in our work.

Although SIR is a widely used treatment for preventing allograft rejection, adverse effects must not be forgotten. In this study, five out of ten animals treated with SIR showed bleeding ulcers as local reactions on the tail after 15-20 days of treatment. Two animals belonging to non-SIR-treated groups showed similar lesions. However, the ulcers of the untreated animals were smaller and healed sooner, suggesting that these lesions may be due to the natural behavior of animals during grooming or their activity in the cage, and the administration of SIR could delay the healing process, or enhance bleeding, and therefore the persistence and enlargement of the lesion. High blood pressure, thrombocytopenia, and wound-healing problems can be found as common side effects of SIR in human renal transplant patients (Hong and Kahan 2000; Zaza et al. 2013), giving support to this hypothesis. Other studies performed with hamsters and SIR

(Laschke et al. 2006) do not describe the appearance of these adverse effects, even though higher doses were administered, but treatments only lasted for two weeks.

SIR seems to be a promising adjuvant for immune therapies. However, there is still long way to go to learn how to control its effects, as some failed attempts to enhance the immune response by using SIR have been described in literature. Chaoul et al. found that the administration of the same dose of SIR used in this work during a therapeutic vaccine protocol in a murine model for lung tumor, impaired the protection achieved by the administration of the vaccine itself (Chaoul et al. 2015). It has also been described in prophylactic approaches, where mice previously treated with SIR were not able to reject a melanoma and breast tumor challenge (Berezhnoy and Castro 2014), but in this case, no vaccine was co-administered.

In conclusion, the co-administration of SIR efficiently potentiated the partial protection conferred by the DNA vaccine carrying the *Leishmania* genes *TRYP*, *PAPLE22*, *KMPII* and *LACK* in the pVAX vector assayed in this study. However, our current data are not enough to demonstrate the basis of the achieved potentiation, suggesting the involvement of a regulated IFN-γ production and antibody intervention. More analyses are needed to elucidate this mechanism.

CONCLUSIONS

1st objective: "To test *in vitro* the efficacy of drugs acting on bioenergetic pathways in the management of visceral leishmaniasis, American and African trypanosomiasis, and malaria"

1- DCA is the only bioenergetic modulator we have found to have anti-parasitic activity at the asexual replicative stages within the host of the three genera of parasites studied. It reduces the parasite burden of infected cells in trypanosomatids and the number of infected cells in *L. infantum* in a dose-dependent manner. Our results show a dose-dependent inhibition of parasite growth by DCA on the intra-erythrocytic stage of *P. falciparum*, albeit no PDK genetic sequences have been identified yet in this parasite.

2- The glycolysis inhibitor 3BP showed anti-parasitic activity on *T. brucei* bloodstream forms, *T. cruzi* and *L. infantum* intracellular amastigotes, with potential for VL and American and African trypanosomiasis treatment. No efficacy of 3BP against *P. falciparum* asexual replicative forms has been found.

3- LND and 2DG showed parasite-killing properties on *T. cruzi* and *P. falciparum*. Antiplasmodial activity of LND has been demonstrated for the first time in this study. In the case of *T. brucei* bloodstream forms, only LND was shown to be efficient. Both *Leishmania* promastigote and amastigote growth were affected by these compounds.

4- MET was shown to be efficient on *in vitro* cultures of *P. falciparum,* and we report, for the first time, its efficacy against *T. cruzi* and *T. brucei*. No effect of this compound was recorded for *Leishmania* amastigotes.

5- SIR did not affect the viability of intracellular *T. cruzi* and *L. infantum*. In fact, a slight increase in parasite load was observed. However, low doses of this compound were needed to achieve a dose-dependent effect on *P. falciparum* and *T. brucei* viability.

2nd objective: "To study the efficacy of bioenergetic modulators selected from the previous chapter in *in vivo* models of visceral leishmaniasis, American and African trypanosomiasis, and malaria"

6- Neither DCA nor 2DG controlled the progression of *P.yoelii* in the *in vivo* model of murine malaria, and no extended life span was detected.

7- No parasite burden control was detected after DCA, MET or 3BP administration to mice models of American and African trypanosomiases.

8- The use of DCA and 3BP in the hamster model of *L. infantum* visceral leishmaniasis did not reduce parasite burdens in main target organs. However, glycolysis inhibitor drugs reduced levels of pro- and counter-inflammatory cytokines, as well as other immune-related parameters, such as immunoglobulin G levels and arginase activity, even in the presence of high parasite burdens in target tissues of animals treated.

3rd objective: "To determine if the administration of sirolimus during the immunization with a DNA vaccine may protect against experimental challenge with *L. infantum*"

9- The DNA vaccine carrying the *Leishmania* genes *TRYP*, *PAPLE22*, *KMPII* and *LACK* in the pVAX vector assayed in this study significantly reduced parasite burdens in skin and lymph nodes of challenged hamsters.

10- The administration of SIR during the expansion and contraction phase of the immune response triggered by the DNA vaccine designed in our laboratory expanded the achieved protection by the vaccine itself, and efficiently reduced parasite burden in the spleen of challenged hamsters. Thus, sirolimus constitutes a good candidate for vaccine boosting in the hamster model of VL.

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