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Leishmania Proteomics: an in silico perspective †

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Abstract

We report on the state of the art of proteins recognized as potential targets for the development of leishmania treatments through the search of biologically active chemical species, either from experimental *in vitro*, *in vivo*, or *in silico* sources. We classify the gathered information, in several ways: vector taxonomy and geographical distribution, leishmania parasite taxonomic and geographical distribution and enzymatic function (oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases and cytokines). Our aim is to provide a much needed reference layout for research efforts aimed to understand the background of ligand-protein activation/inactivation processes, in this specific case, related with enzymes known to be part of biochemical cascade reactions initiated following a leishmania infectious episode.

Introduction

Leishmaniasis is a tropical and subtropical group of zoonotic diseases, caused for different species of *Leishmania* genus.[1,2] It mainly affects mammalians and is transmitted through

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the bite of infected female sand flies.[3,4] Sand fly taxonomic classification is presented in Figure 1. Actual molecular and phylogenetics analysis have allowed to build a rich and complex taxonomic classification of *Leishmania* genus.[2,5] The division of *Leishmania* genus in euleishmania and paraleishmania was proposed as the result of molecular analysis.[6] Euleishmania involves subgenus *L. (Viannia)*, *L. (Leishmania)* and *L. (Sauroleishmania)* (Fig. 2).[6–9] Paraleishmania includes *L. (Endotrypanum)* subgenus, containing only *E. schaudinni* and *E. monterogeii* species (See Fig. 2).[10–13] Colombia have reports of *L. amazonensis*, *L. braziliensis*, *L. mexicana*, *L. colombiensis*, *L. guyanensis*, *L. panamensis*, *L. chagasi*, *L. lainsoni* and *L. equatoriensis* as parasites of leishmaniasis transmitters.[14,15]

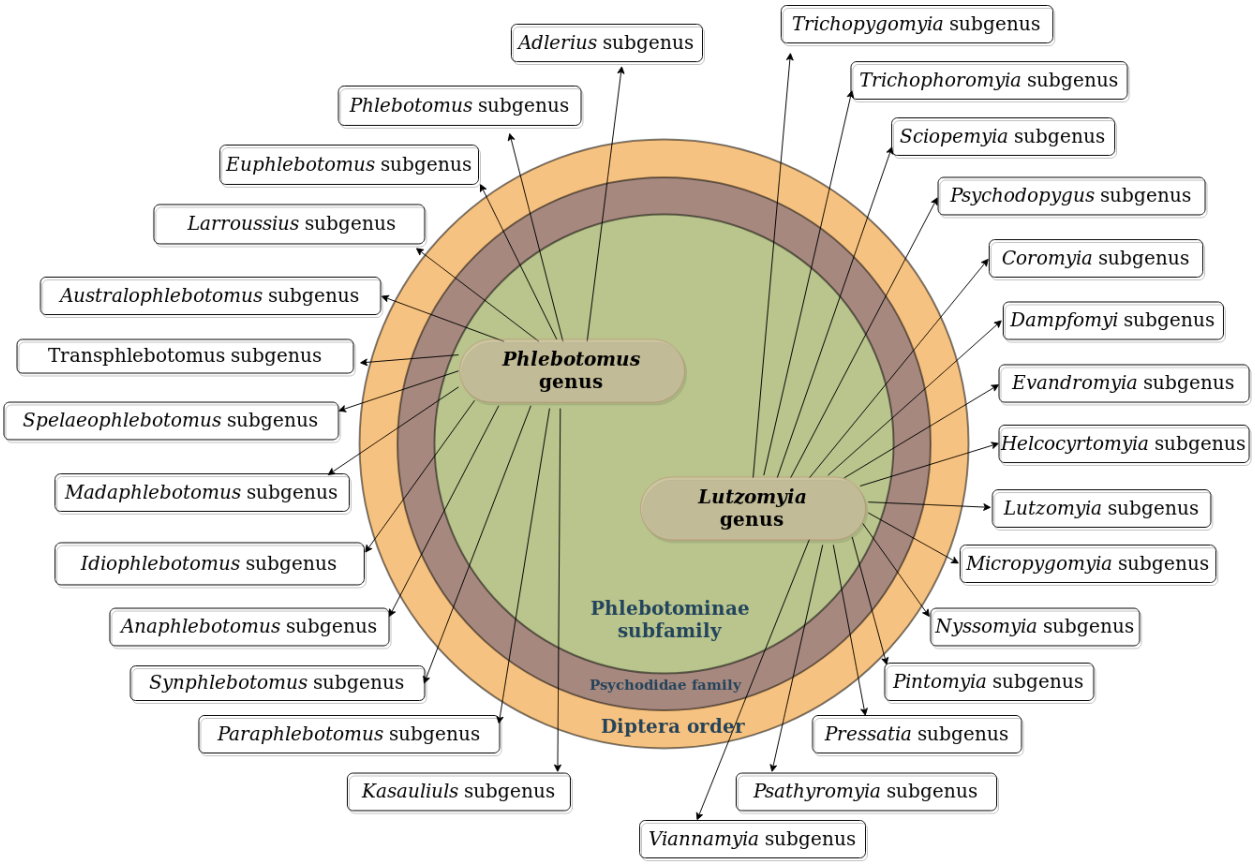

Figure 1: *Leishmania* vector taxonomic and classification and geographical distribution: Diptera order, Psychodidae family, phlebotomiae subfamily, and, geographically, *Phlebotomus* genus in the old world and *Lutzomyia* genus for the new world.[2,11]

The parasitization process involves a myriad of proteic agents, all of them playing preponderant roles in the development of the disease. Several proteins have been recognized as pos-

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sible targets for the development of treatments.[16] Currently, the first line drugs used to treat the disease are: Meglumine antimoniate (Glucantime), Sodium stibogluconate (Pentostam), Amphotericin B (Fungizone), Liposomal amphotericin B (AmBiosome), paromomycin and pentamidine.[16–18] Theses drug are not completely effective against *Leishmania*. There are many research efforts directed towards the development of new bioactive principles to control the disease, many of which turn to natural products (metabolites).[19,20] as a source of inspiration

In this work, we set our goal as to shed light on the the relationship between nature-inspired bioactive chemical structures and macromolecular proteic agents involved in any of the differents levels of the leishmania parasitization process, trough the microscopic-atomistic lense of ab-initio and force-field based models, that is, to use hybrid *in silico* methodologies to model and simulate ligand-protein systems.

Leishmania life cycle and host immune response

Leishmania parasites invade, develop and replicate inside the host Mononuclear Phagocyte System (MPS), attacking macrophages and dendritic cells.[21–23] Incubation time of Leishmania parasite from promastigote to amastigote takes between two and three months, time range when the host immune system response activates and can be favorable or unfavorable.[22] The parasite cycle begins with the bite of an infected female sandfly, carrier of promastigote *Leishmania* parasites in a meta-cyclic state (see Fig. 3).[24,25] At this stage, neutrophils and macrophages are the first line immune cells activated, being neutrophils the initiators of the inflammatory process.[25,26]

Leishmania parasite has two ways to enter the macrophage: A direct path, via the macrophage, and an indirect path, by attacking the neutrophils.[27] The direct path, occurs when the promastigote is directly endocited by the macrophage phagosome, or parasitophorous vacuole, which undergoes a biochemical transformation into phagolysosome.[28]

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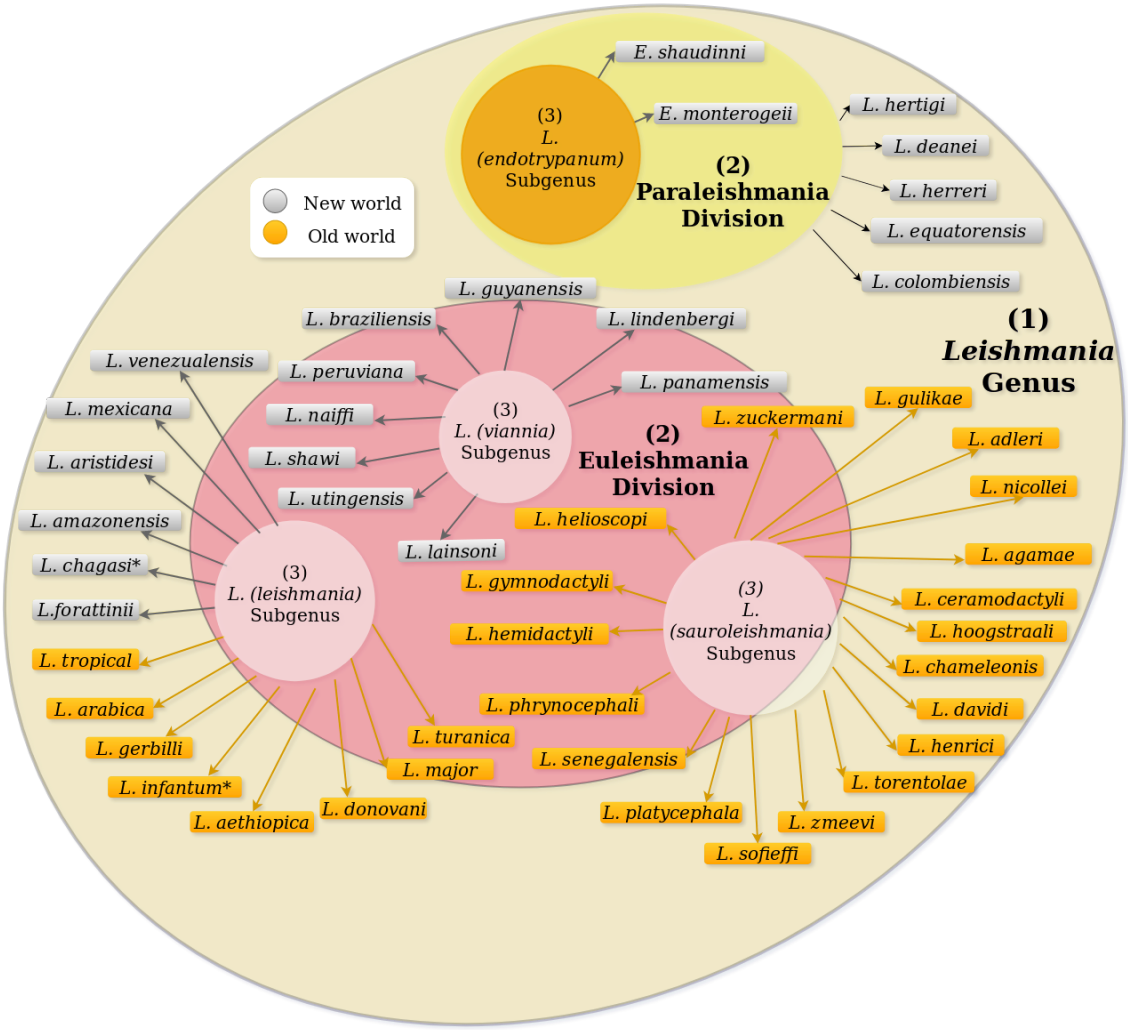

Figure 2: *Leishmania* taxonomic classification and geographical distribution. *Leishmania* genus: euleishmania and paraleishmania. Paraleishmania is conformed by *L. (endotrypanum)* subgenus and euleishmania by *L. (viannia)*, *L. (leishmania)* and *L. (sauroleishmania)* subgenus.[6–9]

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The indirect path goes throug the neutrophil mediated phagocytosis of the parasite, followed by a subsequent macrophage phagocytosis step (see Fig. 3.[27] *Leishmania* promastigote parasite survives inside the phagolysosome vacuole by producing Lipophosphoglycan (LPG), gp63 protein and glutathione transferase.[24,29]

Following the initial macrophage attack, the biochemical response of the host immune system focus on the production of cytokines by specialized T_H $CD4^+$ cells, either T_H1 or T_H2. Among the T_H1 secreted cytokines we found gamma interferon (IFN-γ), which activates and stimulates the macrophages, increasing its microbiocide activity. Interleucine (IL)-12 and IL-2, cytokines also secreted by T_H1 cells, help T_H $CD4^+$ transformation into T_H1, which is the more suitable form to respond to the exogenous attack, in our case, the metacyclic promastigote form of *Leishmania*.[30,31]

Immune T_H2 response produces IL-4, IL-5, IL-10, Transforming Growth Factor Beta (TGF-β), among other cytokines. These compound are more effective facing allergenic diseases or helmintic infections, and therefore is more desirable the T_H1 response. Moreover, the T_H2 response inhibits the T_H1, favoring the propagation and survival of the *Leishmania* parasite.[30,32]

Leishmania protein targets

Crystal structures were retrieved from the Protein Data Bank (PDB) and classified accordingly.[33] The PDB search process was carried using the keywords "*Leishmaniasis*" and "*Leishmania*", providing 338 items, after which we classified the selected proteins according to the parasitization cycle. Other selection criteria considered were: proteins with different PDB-codes but same structure and proteins with equal structures but elucidated from different organisms were considered. An example of proteins that belong to different groups are: Dihydrofolate Reductase-Thymidylate Synthase (DHFR-TS) (PDB ID: 3INV), which is an oxidoreductase and a transferase. A total of 49 proteins comprised the final study

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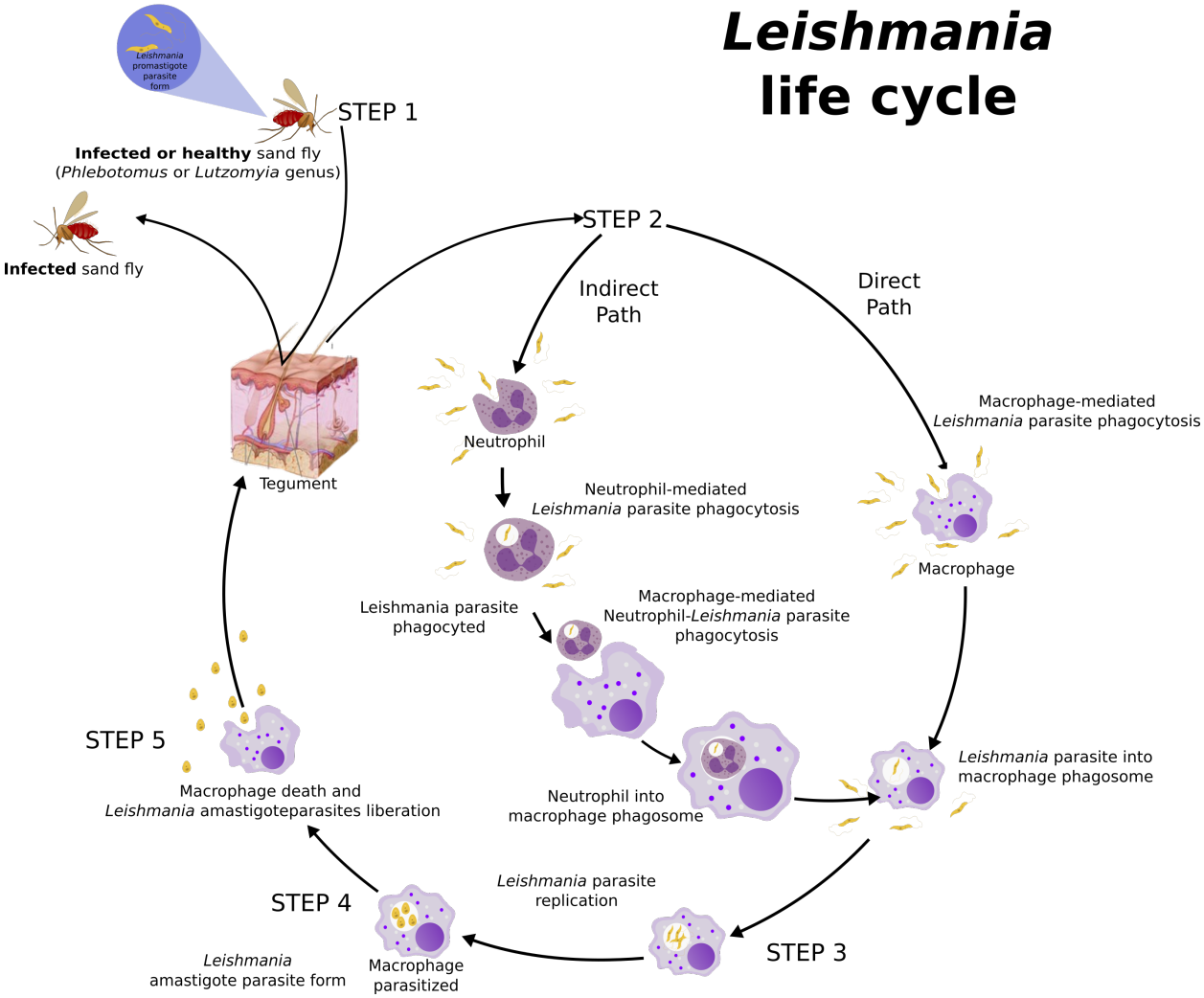

Figure 3: *Leishmania* life cycle can be divided in five steps. **Step 1**: Virulent metacyclic promastigotes are egested when a female sandfly carrier bites a possible host. **Step 2**: Here there are two possible paths. Direct path: promastigotes are phagocytized by macrophage cells. Indirect path: promastigotes are phagocytized by neutrophil cells, subsequently, neutrophils are phagocytized by macrophage. At the end of step 2, promastigotes end up inside a macrophage phagolysosome. **Step 3**: The promastigote parasite produces glutathione transferase, a protein that protects it from the acidic conditions of the phagolysosome, then initiating the replication process . **Step 4**: Transformation of vector promastigote into amastigote takes place. *Leishmania* amastigotes are experts at exploiting host cell machinery to thrive. **Step 5**: Amastigotes are then taken out when a sand fly, either infected or not, bites the host, closing the cycle.

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population as possible drug-targets (see Fig. 4).

From the selected proteins, the most abundant group belongs to oxidoreductases (Enzyme Commission Number - EC 1) and transferases (EC 2), with 15 subjects each one, 6 hydrolases (EC 3), 5 lyases (EC 4), 3 isomerases (EC 5), 3 ligases (EC 6) and one cytokine was found too (see Fig. 4).

Oxidoreductases (EC 1)

This group is comprised by enzymes that catalyzes oxidation-reduction reactions.[34] Glyceraldehyde-3-phosphate dehydrogenase *Leishmania major* (PDB ID: 1GYP and 1A7K) belongs to this group. 1GYP is a homotetrameric enzyme of 156 kDa that catalyzes Glyceraldehyde-3-phosphate (GAP) oxidative phosphorylation to 1,3 Bisphosphoglycerate (BPG) in the glycolysis pathway, through the NAD^+ cofactor.[35]

Malate dehydrogenase (PDB ID: 4H7P) participates in the glycolysis process by reducing oxaloacetate (OAA) to malate.[36] Dihydrofolate Reductase-Thymidylate Synthase (DHFR-TS) (PDB ID: 3INV) and Pteridine reductase (PTR1) (PDB ID: 1E92) from *L. major* and *T. cruzi*, respectively, are responsible of pterin salvaging in parasitic trypanosomatids. Specifically, this happens because *Leishmania* parasites are auxotrophics for folate and other pterins required in critical pathways, including nucleic acid and protein biosynthesis.[37–39] Dihydroorotate dehydrogenase (DHODH) (PDB ID: 3GYE) is a flavoprotein enzyme involved in the *de novo* pyrimidine biosynthesis pathway. 3GYE catalyzes (S)-dihydroorotate oxidation to orotate, in a redox reaction. DHODH is located within the cytosol (Class 1) and inside the mitochondria (Class 2) in eukaryotes and some prokaryotes.[40]

Thymine dehydrogenase JBP1 (PDB ID: 2XSE) is essential for J-Base DNA (β-D-glucosyl-hydroxymethyluracil) biosynthesis and maintenance.[41] Tryparedoxin (PDB ID: 3S9F) and Tryparedoxin peroxidase I (PDB ID: 3TUE) (TXN/TXNPx) reduces macrophages-hydroperoxides produced as the infection progresses.[42] These proteins stay in a cytosolic form. The detoxification pathway is essential for parasite survival.[42] Pseudoperoxidase (PP)

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(PDB ID: 5VIA) *L. major* is a heme protein, expressed in humans during pathogen attack.[43] It is a important protein involved in pathogen defense mechanisms against reactive nitrogen species, such as peroxynitrite, produced as host immune response.[43] Peroxidase (PDB ID: 3RIV) is yet another enzyme involved in this metabolic pathway, and is considered as a potential drug specific target.[44]

Enzyme superoxide dismutase - FeSODA (PDB ID: 2F2N) protects the parasite against macrophage toxic radicals. When the amastigote is phagocytized, macrophage cells produce a respiratory burst generating reactive oxygen intermediates, such as hydrogen peroxide, OH^-, $O_2{}^-$ radicals and peroxynitrite, as part of the macrophage mechanism to fight invasive microorganisms.[45–47] Thus, 2F2N acts as the first line of defense against those Reactive Oxygen Species (ROS). This fact, makes 2F2N a suitable enzymatic target for *Leishmania* controlling drug development.[45]

Tryopanothione reductase *Leishmania infantum* (PDB ID: 2YAU) is a key enzyme from the *Leishmania infantum* polyamine-dependent redox metabolism.[48] Prostaglandin F synthase from *L. major* (PGF; PDB ID: 4F40) is involved in the lipid metabolic pathway, acting through a NADP cofactor.[49] Sterol 14α-demethilase (CYP51) *L. infantum* is a enzyme that catalyzes the removal of the 14α-methyl group from sterol precursors. This reaction is essential for membrane cell biosynthesis, specifically, CYP51 it relates to the ergosterol pathway, and is believed to be crucial for the survival of infectious *Leishmania* parasite.[50]

Transferases group (EC. 2)

Transferases catalyze reactions in which a chemical group is transferred from a electron/proton donor substrate to an electron/proton acceptor substrate.[34] These proteins catalyze key cellular processes in all kingdoms of life, such as, DNA repair, RNA editing, and activation of metabolites used in catabolic pathways.[52] Adenine phosphoribosyltransferase (APRT) (PDB ID: 1QB7 and 1MZV) belongs to the phosphoribosyltransferase family type I (PRTs) and is involved in the purine-salvaging process, catalyzing adenines to adenosine-5-monophosphate

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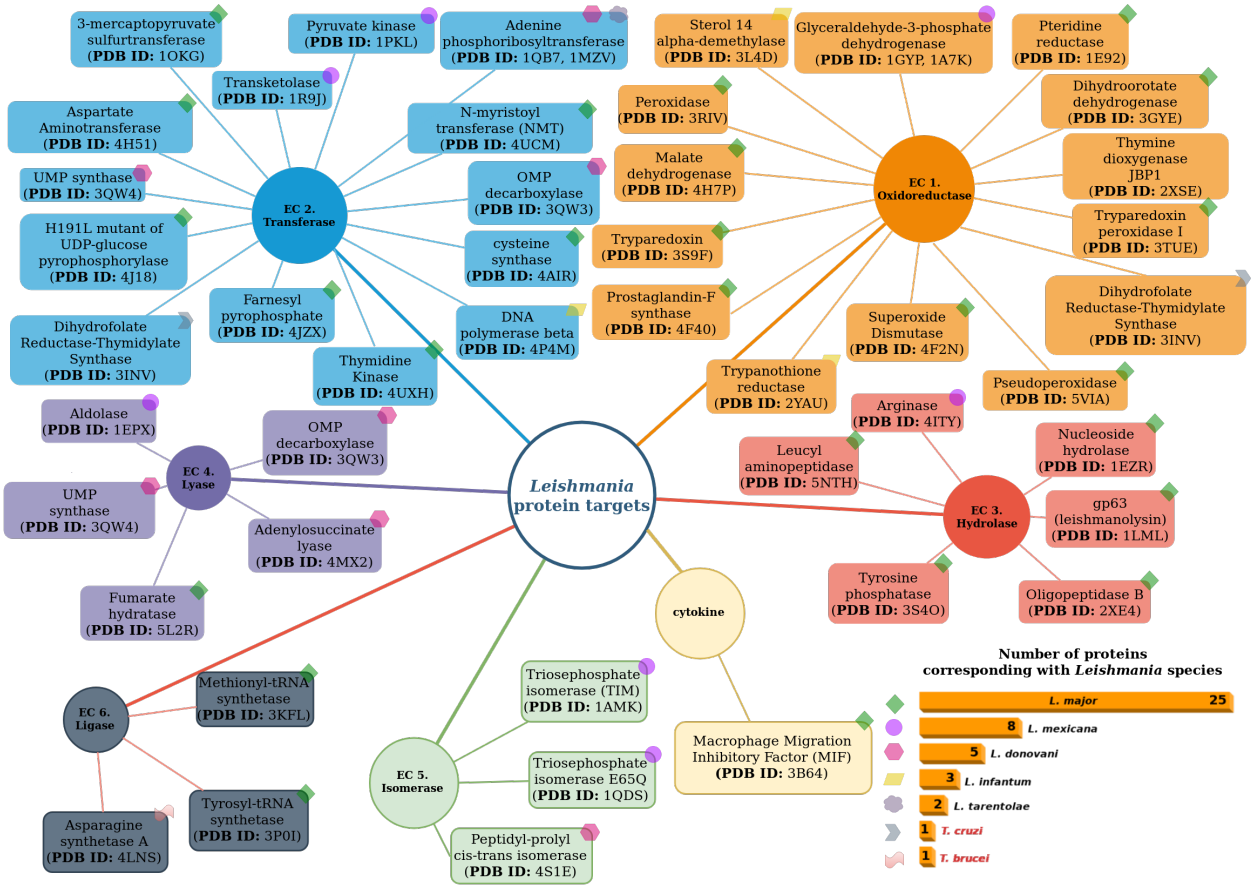

Figure 4: *Leishmania* protein classification. Oxidoreductases (orange), transferases (blue), hydrolases (red), lyases (violet), isomerases (green), ligases (dark blue) and cytokines (yellow). The chart at the bottom right of the figure shows the number of proteins found for each species. *T. cruzi* and *T. brucei* do not belong at the *Leishmania* protein group, but they have been used in some studies as homologous proteins.[39,51]

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(AMP).[53,54] It is a key enzyme, because, protozoan parasites are auxotrophic for the purine *de novo* biosynthesis pathway. N-myristoyltransferase (NMT) (PDB ID: 4UCM) catalyzes the co-translational transfer of myristate from myristoyl-CoA to the N-terminal glycine in a large number of proteins. Modifications have been implicated in localization and/or substrate activation.[55] OMP decarboxylase (PDB ID: 3QW3) and UMP synthase (PDB ID: 3QW4) are two enzyme involved in the last two steps of *de novo* Uridine 5'-monophosphate (UMP) biosynthesis, for pyrimidine production (as opposed to the purine-salvaging pathway).[56] Both enzymes are recognized as possible drug targets.

Cysteine synthase (PDB ID: 4AIR) is involved in cysteine biosynthesis. Cysteine is required for protein biosynthesis in *Leishmania* species, also, it is a source of reduced sulfur for important metabolites, such as coenzyme A (CoA), enzyme cofactors and iron-sulfur clusters.[57] DNA polymerase Beta (PDB ID: 4P4M) is required for maintenance, replication and recombination of the DNA.[58] This protein is specially required for the *Leishmania* parasite amastigote form.[58] Thymidine kinase (PDB ID: 4UXH) catalyzes ATP γ-phosphate transfer to 2'-deoxythymidine (dThd), forming thymidine monophosphate (dTMP). It is a important enzyme because plays a key role in parasitization process.[59] Farnesyl pyrophosphate (FPPS) (PDB ID: 4JZX) is involved in ergosterol synthesis too, acting in the early steps of isoprene synthesis and maintainance of lipid bilayer integrity.[60,61] It is a potential enzymatic target, because, it was successful inhibited with bisphosphonate previously.[60]

UDP-glucose pyrophosphorylase *L. major* (PDB ID: 4J18) catalyzes the reversible conversion of glucose-1-phosphate (Glc-1-P) and uridine 5'-triphosphate (UTP) to UDP-Glc and inorganic pyrophosphate (PPi) in the presence of Mg^{2+}, into glycolytic pathway.[52] Aspartate aminotransferase (AAT) (PDB ID: 4H51) catalyzes reversible transfer of the α-amino group of aspartate and glutamate, converting L-aspartate and 2-oxoglutamate to oxaloacetate and L-glutamate (http://brenda-enzymes.info).[62] This enzyme is pyridoxal phosphate (PLP) cofactor dependent and a potential drug target.[63] 3-mercaptopyruvate sulfurtransferase (PDB ID: 1OKG) is involves in cysteine metabolism, having three domains: N-terminal, core and

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C-terminal. The active site of the protein is localized along the N-terminal and core domains, containing the Cys-253, Arg-74 and 185 as sulfite inhibition amino acids. Asp-61, His-75 and Ser-255 are close to Cys-253, and polarize the carboxyl group of 3-mercaptopyruvate through a tiophilic attack.[64] Transketolase (PDB ID: 1R9J) is a key enzyme in the nonoxidative branch of the pentose phosphate pathway (PPP), which transfers two-carbon glycoaldehyde units from ketose-donors to aldose-acceptor sugars.[65] Finally, pyruvate kinase (PDB ID: 1PKL) catalyzes the phosphoenolpyruvate-phosphate group transfer to adenosine diphosphate. This enzyme is involved in the glycolytic pathway.

Hydrolases group (EC. 3)

Hydrolases catalyze reactions in which a bond in a substrate is hydrolyzed to produce two fragments.[34] Nucleoside hydrolase *L. major* enzyme (PDB ID: 1EZR) is the main responsible of nucleotide salvaging from host, due *Leishmania* parasite lack *de novo* biosynthesis pathway of purines.[66] The *Leishmania* parasite promastigote form, express glycoproteins in its surface; theses enzymes are know as Leishmanolysin (*gp63* gene) (PDB ID: 1LML).[67] It have an important role in macrophages infection process.[67]

Oligopeptidase B (OPB) *L. major* (PDB ID: 2XE4) catalyzes the Arg- and Lys- bond formation in proteins, and is a potential drug target, according to several studies.[68] Tyrosine phosphatase (PRL-1) (PDB ID: 3S4O) is mainly secreted by the *Leishmania* parasite promastigote form, however, the parasite amastigote form produces PRL-1 more efficient and abundantly during macrophage infection process.[69] It is important for the parasite survival. Leucine aminopeptidase (LAP) *L. major* (PDB ID: 5NTH) is involved in N-terminus catalysis of proteins. This protein has multiple functions; in host infection processes it provides essential amino acid, and is a major transcription regulator, among other functions.[70] Finally, the arginase protein (PDB ID: 4ITY) catalyzes the first step polyamine biosynthesis. This process entails cellular growth as well as parasite survival.[71]

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Lyase group (EC. 4)

Group of enzymes that catalyzes non-hydrolytic reactions, in which a chemical group is cleaved and removed from a substrate leaving a double bond.[34] Adenylosuccinate lyase (ASL) (PDB ID: 4MX2) is a lyase protein, and have been identified as vital component of purine salvaging in *Leishmania donovani*.[72] Fumarate hydrolase (FH) class 1 (PDB ID: 5L2R) enzyme is a cytosolic protein with two isoforms: a mitochondrial (Class1) and a cytosolic (Class2). FH class 1 produces fumarate substrate for the dihydroorotate dehydrogenase. Additionally, this enzyme migrate to cellular nucleus, where play a key rol in DNA repair.[73] Finally, aldolase *L. mexicana* (PDB ID: 1EPX) enzyme, is involves in glycolytic pathway and catalyzes the Fructose-1,6-bisphosphate conversion to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate.[74]

Isomerases group (EC. 5)

Isomerase proteins catalyzes one-substrate/one-product reactions that can be regarded as isomerization reactions.[34] Triosephosphate isomerase (TIM) (PDB ID: 1AMK) plays a central rol in the glycolysis process, as catalyst of dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde-3-phosphate (GAP).[75] Currently, there exits a TIM E65Q mutant (PDB ID: 1QDS), being more stable than the TIM wild-type.[76] Peptidyl-prolyl cis-trans isomerase (PDB ID: 4S1E), accelerates the folding process of proteins. It catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides (https://www.uniprot.org/uniprot/Q9U9R3).[77]

Ligases group (EC. 6)

Ligase proteins catalyzes the joining together of two or more molecules coupled to hydrolysis of ATP or an analogous molecule.[34] This group has three proteins classified as possible enzymatic targets. First and second enzyme are Methionyl-tRNA synthetase (PDB ID: 3KFL) and Tyrosyl-tRNA synthetase (PDB ID: 3P0I), which were elucidated with MgATP as substrate and methionine as solvent, and are recognized as essential for biological processes,

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such as gene translation.[78,79] The three enzyme is Asparagine synthetase A (ASNA) (PDB ID: 4LNS), which is an ammonium and glutamine dependent enzyme. In experimental (*in vivo* and *in vitro*) studies, this protein-deletion-gene caused parasite growth delay, therefore is recognized as a possible target.[51]

Cytokines group

The Migration Inhibitory Factor (MIF) from *L. major* (PDB ID: 3B64) was classified as possible enzymatic target. This cytokine is an ortholog of human MIF, also known as Lm1740MIF. 3B64 interacts with MIF receptors, HLA class II histocompatibility antigen gamma chain (also called invariant chain or CD74) and exhibits an antiapoptotic activity that may facilitate the intracellular persistence of *Leishmania* into macrophages.[80]

Leishmaniasis drugs

Pentavalent antimonials (Sb(V)) were firsts leishmaniasis drugs for along time, however, development of *Leishmania* resistance made it drug inefficient. New drug and treatment investigations have been development.[2] Currently, being developed much effective drugs, but it generates secondary effects as high toxicity, also, these treatments and drugs are expensive and generates resistance for *Leishmania* parasites.[81,82]

Leishmaniasis disease is a Neglected Tropical Disease (NTD), therefore, it does not have necessaries economic funds and strict epidemiological controls from governmental organizations, made difficult drugs and treatments development.[83]

Standard drugs

Currently, leishmaniasis is treated using the following types of chemical substances: antimonials (Sb(V)), amphotericin B, Pentamidine, Miltefosine (hexadecylphosphocholine), paromomycin (aminosidine), sitamaquine and pamidronate (see Fig. 5).[17,84] These drugs are used for treats three main leishmaniasis forms: cutaneous leishmaniasis (CL), mucocu-

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taneous leishmaniasis (MCL) and visceral leishmaniasis (VL).[81] Antimonials were the first antileishmania compounds, introduced in the 40s decade.[2,84] They are available as meglumine antimoniate (Glucantime) and sodium stibogluconate (Pentostam), and these are standard first line drugs for treatment, but emergence of resistance has limited their use.[16,82] Antimonials are used for VL treatment, but, different studies found that *L. donovani* and *L. braziliensis* were more sensitive to sodium stibogluconate than *L. major*, *Leishmania tropica* and *L. mexicana*.[2,17] Amphotericin B is a macrolide antibiotic isolated from *Streptomyces nodosus* in 1956 and widely used since the 80s as amphotericin B deoxycholate.[2,84] It selectively inhibits the membrane synthesis of the parasite and causes holes in the membrane, leading to parasite death.[84] It is used as a second-line treatment, and is present in five formulations: amphotericin B deoxycholate (Fungizone), Emulsification of Fungizone in intralipid 20 %, liposomal amphotericin B (AmBisome), amphotericin B lipid complex (ABLC; Abelcet) and amphotericin B cholesterol dispersion (ABCD; Amphotec) (see Fig. 5).[17,84] Amphotericin B and its lipid formulations are used as alternative chemotherapeutic treatments.[16] Lipid formulations of amphotericin B have been gaining importance, becoming the fist-choice established leishmaniasis treatment by the US Food and Drug Administration (FDA).[2]

Pentamidine is widely recognised, its antileishmanial activity is on *Leishmania* parasite polyamines biosynthesis and mitochondrial membrane. They are considered second-line treatment drugs for VL, because its toxicity (myalgia, nausea, headache, hypoglycemia, irreversible insulin dependent diabetes mellitus and death), declining its efficacy. Also, its hig2 monetary costs renders them prohibitive.[84] India and East Africa used paromomycin as a cheap alternative treatment, despite its toxicity.[2] Paromomycin remained neglected until the 80s, when topical formulations for VL were developed.[84] One World Health, the Bill and Melinda Gates Foundation, Gland Pharma Limited, IDA Solutions and WHO/TDR partnered to develop Paromomycin as a public health tool to be sold on a not-for-profit basis, at a very low price.[84] Miltefosine, initially developed as an anticancer drug, currently is the first

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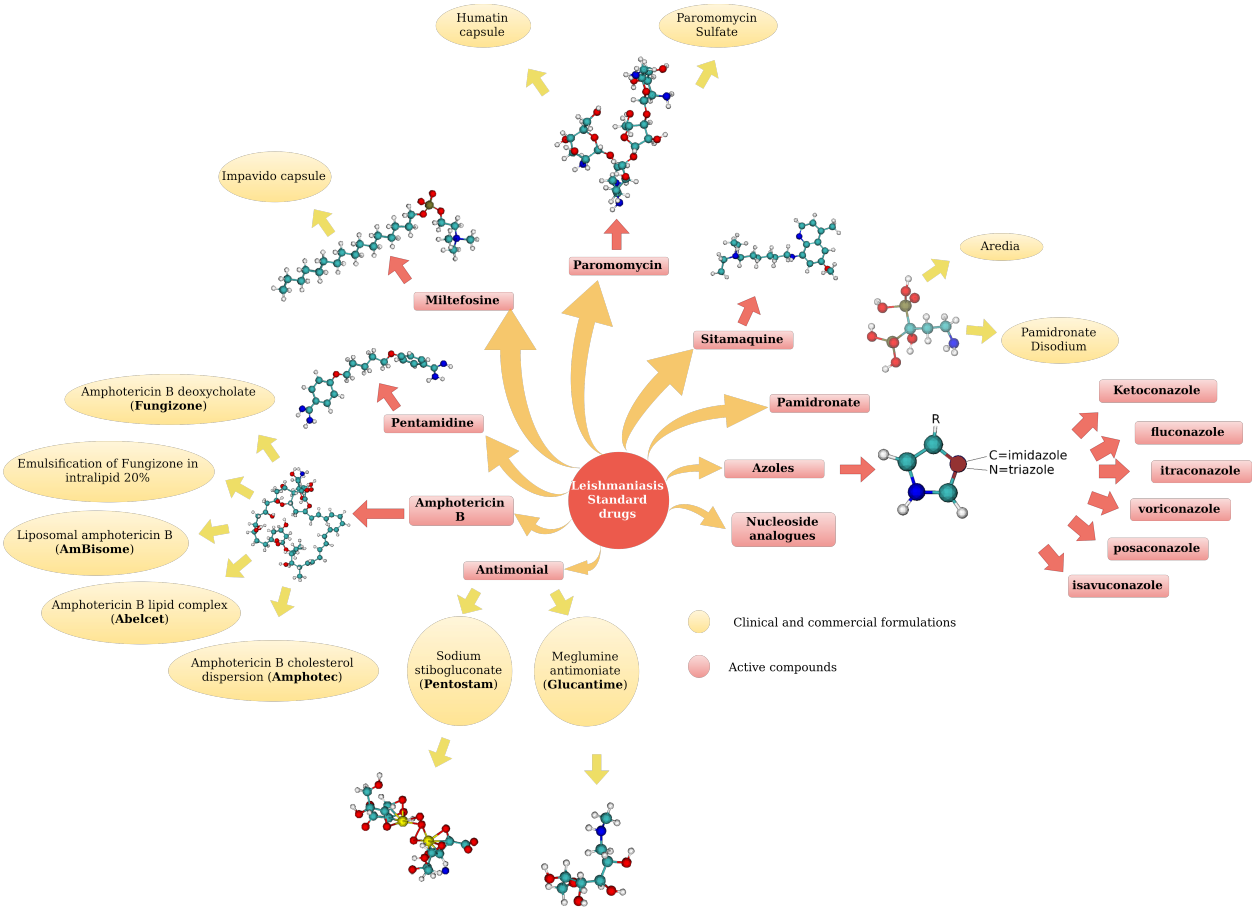

Figure 5: Drugs used against leishmaniasis can be divided in two main groups: standard drugs and alternative drugs (metabolites). Graphics show molecular structures of drug active compounds. Structures were obtained from ChemSpider (http://www.chemspider.com/).

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effective oral treatment of VL.[2,84] Variation in the sensitivities of both, promastigote and amastigote stages of *L. donovani*, *L. major*, *L. tropica*, *Leishmania aethiopica*, *L. mexicana* and *L. panamensis*, were investigated *in vitro*. From these assays, *L. donovani* was recognized as the most sensitive species to this treatment.[17] Miltefosine ED_50 against *L. donovani* was measured in the range of 0.12 to 1.32 μM. Sitamaquine is rapidly metabolized, forming desethyl and 4-CH2OH derivatives, which might be responsible for its activity. Toxicity appears to be relatively mild, as it causes mild methemglobinaemia.[84,85] Finally, pamidronate is a bisphosphonate drug with significant activity against *Leishmania donovani in vitro*. FPPS protein (PDB ID: 4JZX) is potently inhibited by bisphosphonates in the trypanosomatid parasite.[84] Other two type of drugs considered for leishmaniasis treatment are azoles and nucleoside analogues.[17,86] Within the azole group are, for example, ketoconazole and itraconazole, which inhibits the C14α-demethylase. Nucleoside Analogues, such as allopurinol and pyrazolopyrimidine, are known to inhibit enzymatic processes of the purine salvaging pathway in Leishmania.[17]

Non standard drugs: plant metabolites

Leishmania resistance against antileishmanial drugs suggests that the current use of monotherapies has to be reviewed. The rationale behind combination therapies is to prevent the risk of parasite resistance, provide increased efficacy resulting from synergistic effects, lower dose requirements and reduce duration of therapy eventually leading to reduced toxic effects of the drugs.[81] Natural compounds, such as secondary plant metabolites, are another type of alternative treatments. We retrieved information of 222 compounds isolated of plants that have antileishmanial activity (Anexo 1). The search process was performed through revision of the state of the art.[19,20,87] These compounds were isolated from several parts of the plants, and proved in experimental assays (*in vitro* and *in vivo*) under different physical chemistry conditions and *Leishmania* species.

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