 doi:10.20944/preprints201902.0122.v3

Leishmania Proteomics: an in silico perspective †

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Abstract

We report on the state of the art of scientific literature about 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, 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 underpinning physical interactions in ligand-protein activation/inactivation processes. In the specific case of *Leishmania*, we focus on enzymes known to be part of the biochemical molecular processes initiated following a *Leishmania* infectious episode.

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Introduction

Leishmaniasis is a tropical and subtropical group of zoonotic diseases, caused for species of *Leishmania* genus.[1,2] It mainly affects mammalians and is transmitted through the bite of infected female sandflies.[3,4] Actual molecular and phylogenetics analysis have allowed to build a rich and complex taxonomic classification of *Leishmania* species.[2,5] First, were proposed the division of *Leishmania* genus in Euleishmania and Paraleishmania 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]

In this work, we set our goal to shed light on the the relationship between nature-inspired bioactive chemical structures and proteic agents involved in any of the steps of the *Leishmania* parasitization process, through the microscopic-atomistic lens of *ab-initio* and force-field machanical-statistical based models, that is, to use hybrid *in silico* methodologies to model and simulate ligand-protein systems.

Leishmania vectors

Sandflies are parasitic vectors for dangerous diseases. They can even end up with the host death.[16]

Between range of infected agents from sandfly vectors have *Leishmania* parasites, *Bartonella bacilliformis* bacteria and some viruses.[16–18]

These sandflies required a suitable ecological habitat to survive, such as rural and peri-urban areas, that is, zones with abundant vegetation and little intervention of anthropic activities.[16] Also, sandfly environment are linked with specific climatic, temperature and hu-

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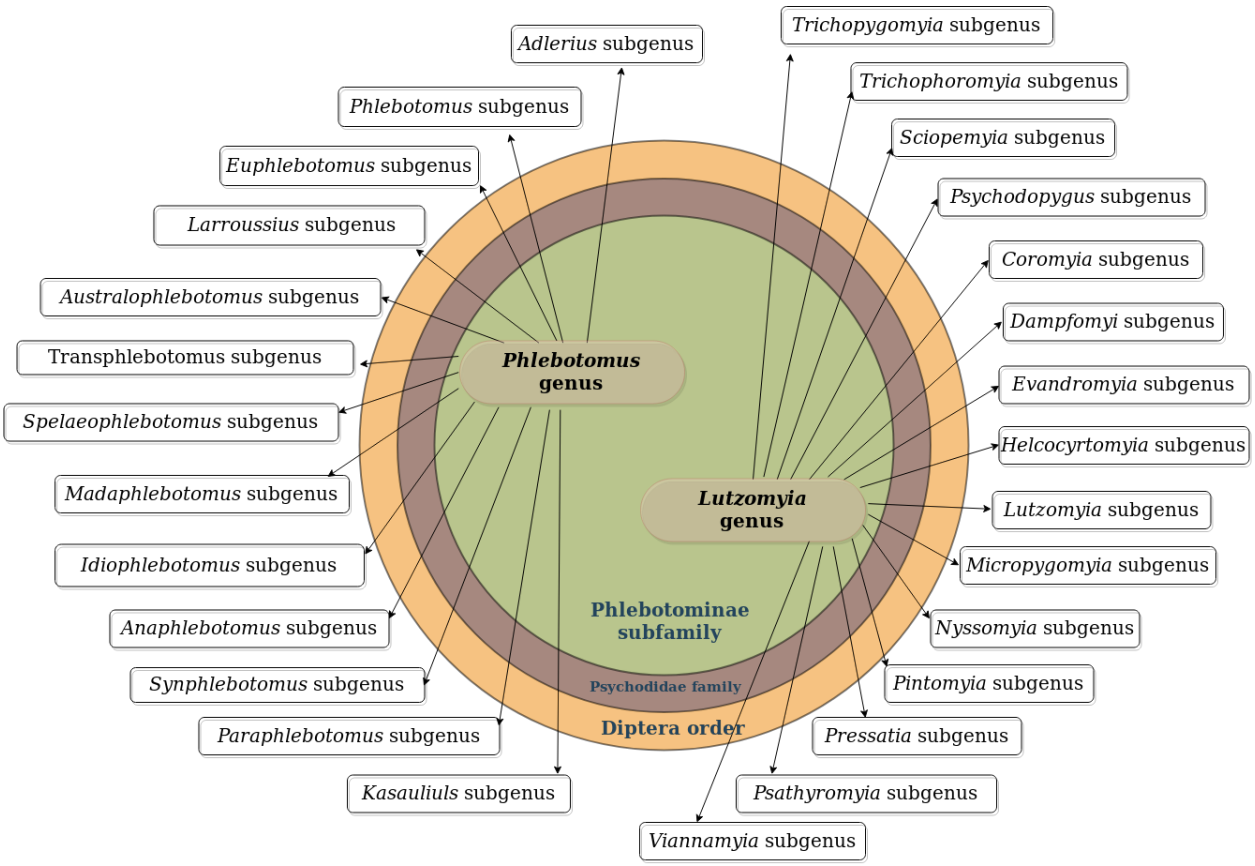

Figure 1: *Leishmania* vector taxonomic and classification and geographical distribution: Diptera order, Psychodidae family, phlebotomiae subfamily, and, geographically, *Phlebotomus* genus in the old world and *Lutzomyia* genus for the new world.[2,11]

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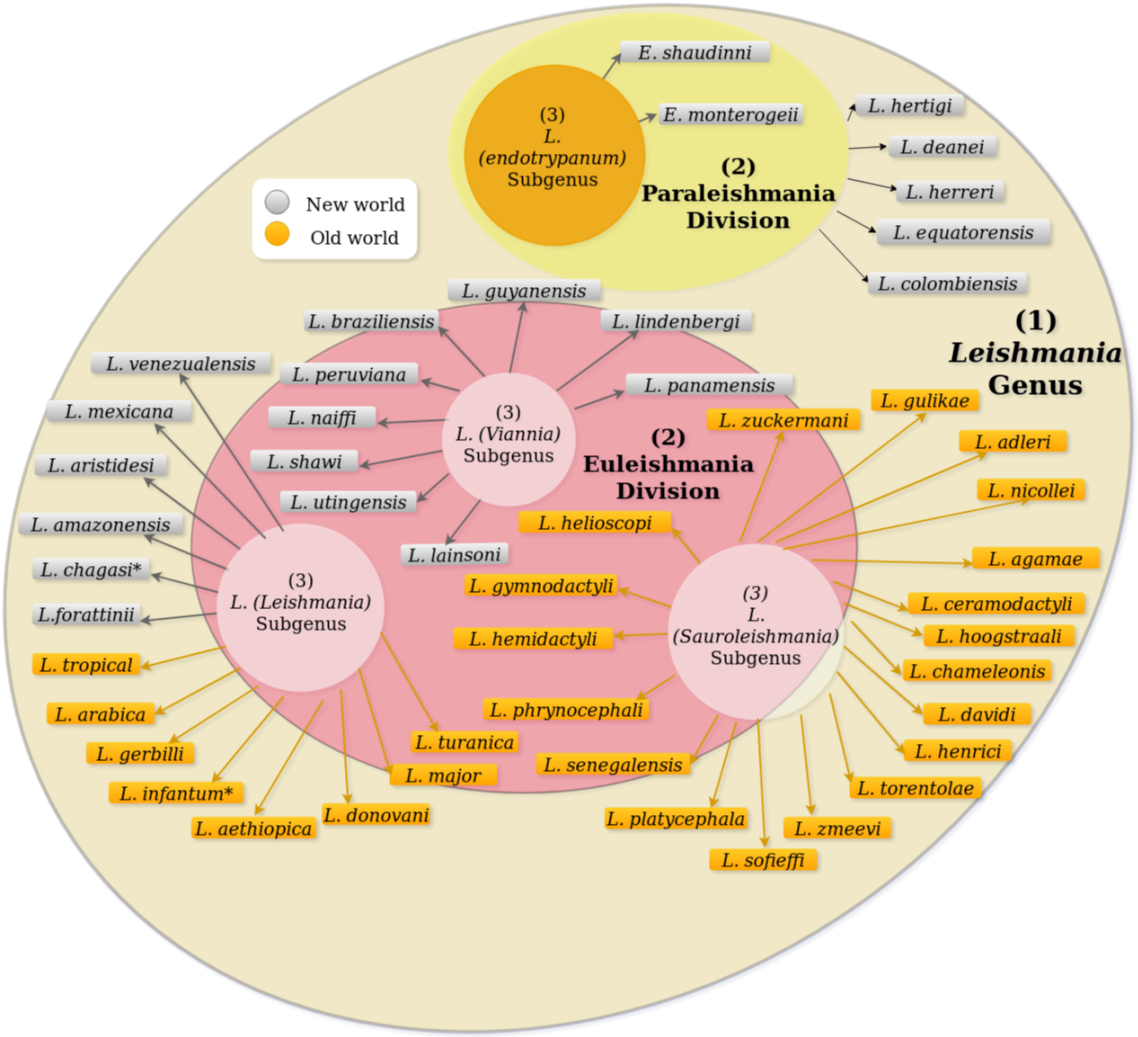

Figure 2: *Leishmania* taxonomic classification and geographical distribution. *Leishmania* genus: euleishmania and paraleishmania. Paraleishmania is conformed by *L. (endotrypanum)* subgenus and euleishmania by *L. (viannia)*, *L. (leishmania)* and *L. (sauroleishmania)* subgenus.[6–9]

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midity conditions.[16,19] Sandfly hematophagous activity is frequently in the night and areas with low light[19]

Sandfly species reported belong to Diptera order into Psychodidae family and Phlebotominae subfamily, these are 900 species approximately divided in five genus. *Phlebotomus*, *Sergentomyia* are prevalent in the old world and *Lutzomyia*, *Brumptomyia* and *Warileya* in the new world.[20–22] *Leishmania* parasites are only transmitted through *Phlebotomus* and *Lutzomyia* genus sandfly vectors.[20] (Ver figura 1). Colombia have reported nine species belong to *Leishmania* genus and 14 species belong to *Lutzomyia* genus.[15,23] Parasite species Colombia reported are *L. amazonensis*, *L. braziliensis*, *L. mexicana*, *L. colombiensis*, *L. guyanensis L. panamensis*, *L. infantum*, *L. lainsoni* and *L. equatoriensis* (ver Fig. 2). *Lutzomyia* species Colombia reported are *L. flaviscutellata*, *L. colombiana*, *L. spinicrassa*, *L. pia*, *L. towsendi*, *L. hartmanni*, *L. umbratilis*, *L. longiflocosa*, *L. trapidoi*, *L. panamensis*, *L. yuli yuli*, *L. cruciata*, *L. columbiana* and *L. gomezi.*[23]

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.[24–26] 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 leads to an favorable or unfavorable outcome.[25] 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).[27,28] At this stage, neutrophils and macrophages are the first line immune cells activated, being neutrophils the initiators of the inflammatory process.[28,29]

Leishmania parasite has two ways to enter the macrophage: A direct path, via the macrophage, and an indirect path, by attacking the neutrophils.[30] The direct path, occurs when the promastigote is directly endocited by the macrophage phagosome, or para-

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sitophorous vacuole, which undergoes a biochemical transformation into phagolysosome.[31] The indirect path goes through the neutrophil mediated phagocytosis of the parasite, followed by a subsequent macrophage phagocytosis step (see Fig. 3.[30] *Leishmania* promastigote parasite survives inside the phagolysosome vacuole by producing Lipophosphoglycan (LPG), gp63 protein and glutathione transferase.[27,32]

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*.[33,34]).

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.[33,35]

Leishmania protein targets

Crystal structures were retrieved from the Protein Data Bank (PDB).[36] The PDB search process was carried using the keywords "*Leishmaniasis*" and "*Leishmania*", providing 425 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

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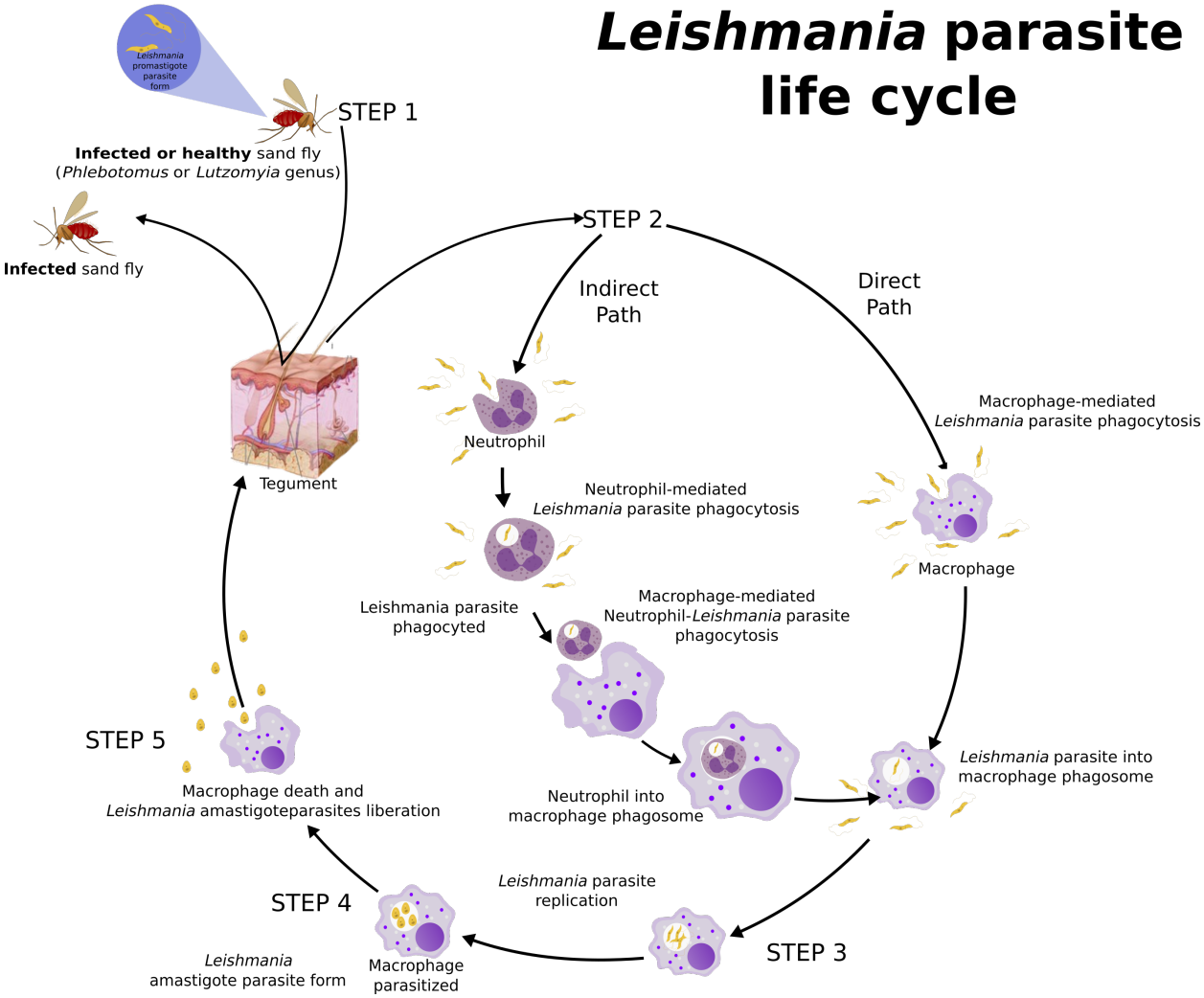

Figure 3: *Leishmania* life cycle can be divided in five steps. **Step 1**: Virulent metacyclic promastigotes are egested when a female sandfly carrier bites a possible host. **Step 2**: Here there are two possible paths. Direct path: promastigotes are phagocytized by macrophage cells. Indirect path: 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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and a transferase. A total of 49 proteins comprised the final study population as possible drug-targets (see Fig. 4).

From the selected proteins, the richest 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), one cytokine and one Lipid Binding Protein (see Fig. 4).

Oxidoreductases (EC 1)

This group is composed by oxidation-reduction enzymes that catalyse reactions in which a substrate donate one or more electrons to an electron acceptor, becoming oxidized in the process.[37] The glycolysis and Pentose Phosphate Pathways (PPP) are a key component of cellular metabolism in Trypanosomatids and these are depended of several oxidoreductase enzymes.[38] In these processes, glucose and other hexoses are critical cellular nutrients to *Leishmania* parasites, where these parasites (promastigote or amastigote life form) are able to import sugar from the extracellular environment or synthesize *de novo*, via gluconeogenesis.[39] Promastigote parasites made both process, but amastigote parasites only carry out gluconeogenesis process.[39]

Glyceraldehyde 3-phosphate dehydrogenase *Leishmania major* (PDB ID: 1GYP and 1A7K, see Fig. 4) belongs to oxidoreductase group and participate in Pentose Phosphate and glycolysis metabolic pathways.[38] This is a key glycolytic homotetrameric enzyme of 156 kDa that catalyzes the conversion of glyceraldehyde 3-phosphate (G3P) into 1,3-biphosphoglycerate (1,3-BPG) with reduction of NAD^+ to NADH, through the NAD^+ cofactor.[40,41] Malate dehydrogenase (PDB ID: 4H7P) participates in the gluconeogenic process by conversion of oxaloacetate (OAA) and malate, used NAD/NADH coenzyme system.[42,43] 4H7P have two isoforms in eukaryotes, differing in their subcellular localization and their specifity for the coenzyme NAD (all types of malate dehydrogenases) or NADP (only malate dehydrogenases from chloroplast cells).[43]

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Dihydrofolate Reductase-Thymidylate Synthase (DHFR-TS) (PDB ID: 3INV) and Pteridine reductase (PTR1) (PDB ID: 1E92) elucidated from *L. major* and *T. cruzi*, respectively, are responsible of pterin salvaging in parasites belonging to Trypanosomatidae family. These salvage pathways are need for normal metabolic processes in *Leishmania* parasites, because theses microorganism are auxotrophics for folate compounds which are required in critical *Leishmania* metabolic pathways, including nucleic acid and protein biosynthesis.[44,45] The DHFR enzyme structure between hosts and parasites diverged extensively, which has permitted the synthesis of several specific DHFR inhibitors known as antifolates.[46] Dihydroorotate dehydrogenase (DHODH) (PDB ID: 3GYE) is a flavoprotein enzyme involved in the *de novo* pyrimidine biosynthesis pathway with other six catalytic proteins.[47,48] The pyrimidine biosynthesis pathway in *Leishmania* parasites is important for DNA and RNA biosynthesis, protein glycosylation, membrane lipid biosynthesis and strand break repair.[47] The DHODH enzyme are divided in the major class 1 (A and B) and 2, and this division are correlates with subcellular location of the protein.[49,50] The class 1 proteins have cytosol subcellular location and are found in Gram-positive bacteria, in the anaerobic yeast *Saccharomyces cerevisiae* and in all trypanosomatids species.[47,50] The 3GYE protein belong to class 1, and catalyzes (S)-dihydroorotate oxidation to orotate in a redox reaction.[50]

Eukaryotic unicellular kinetoplastid flagellates, such as *Trypanosoma* and *Leishmania* species, contain a unique hypermodified base in their nuclear DNA, called J base or β-D-glucosylhydroxymethyl-uracil.[51–53] J-base is associated with the silencing of telomeric expression sites for the variant surface glycoprotein (VSG) genes, but not in actively transcribed VSG genes.[51,52] Currently, it know that the JBP1 protein is required to J-Base biosynthesis and maintenance, also, this enzyme is indispensable for *Leishmania* parasites growth and survival.[54,55] Specifically, JBP1 have the DNA-Binding JBP1 domain (DB-JBP1) (PDB ID: 2XSE) that binds to J-DNA and it is a potential drug target.[54,56]

Macrophages defense mechanism against *Leishmania* parasites, produce peroxynitrite, hydroxyl radicals, hydrogen peroxide, hydroperoxide, superoxide radicals species, among

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other.[57–59] These compounds are toxic to *Leishmania* parasite metabolism and affect its survival, but these parasites have a trypanothione mediated hydroperoxide metabolism to eliminate endogenous or exogenous oxidative agents.[60] Tryparedoxin (PDB ID: 3S9F) and Tryparedoxin peroxidase I (PDB ID: 3TUE) (TXN/TXNPx) protein system reduces macrophages-hydroperoxides species to water, produced during the infection progresses.[61] These proteins stay in a cytosolic form and act on the detoxification pathway as essential process for parasite survival.[61] Pseudoperoxidase *L. major* (*Lm*PP) (PDB ID: 5VIA) is a heme protein expressed by *Leishmania* parasites against Reactive Nitrogen Species (RNS), which have the ability to detoxify RNS.[62,63] The Heme peroxidases utilize peroxides to oxidize a variety of physiologically important molecules.[62] In this case, the enzyme in study is ascorbate peroxidase (APX) (PDB ID: 3RIV), which is considered as a potential drug target.

Superoxide dismutase enzyme (FeSODA) (PDB ID: 4F2N) protects the *Leishmania* parasite against macrophage toxic radicals. When the amastigote is phagocytized, macrophage cells produce a respiratory burst generating ROS intermediates, such as hydrogen peroxide, OH^-, $O_2{}^-$ radicals and peroxynitrite, as part of the macrophage mechanism to fight invasive microorganisms.[64–66] Thus, 4F2N acts as the first line of defense against those ROS. This fact, makes 2F2N a suitable enzymatic target for *Leishmania* controlling drug development.[64] Tryopanothione reductase (TR) (PDB ID: 2YAU) is homodimeric enzyme extracted from *Leishmania infantum* and it is essential for parasite survival.[67] This enzyme catalyse the reduction of trypanothione by NADPH, protecting the parasite against oxidative damage and toxic heavy metals.[67–69] TR reduces trypanothione disulfide (TS_2), a bis (γ-L-glutamyl-L-cysteinylglycine) spermidine or bis (glutathionyl) spermidine conjugate, to the di-thiol form [$T(SH)_2$].[68] The enzyme 9,11-endoperoxide prostaglandin H2 reductase from *L. major* (PDB ID: 4F40) is involved in the lipid metabolic pathway, acting through a NADP cofactor.[70,71] Sterol 14α-demethilase (CYP51) *L. infantum* (PDB ID: 3L4D) 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 relates to the ergosterol pathway, and is

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believed to be decisive for the survival of infectious *Leishmania* parasite.[72]

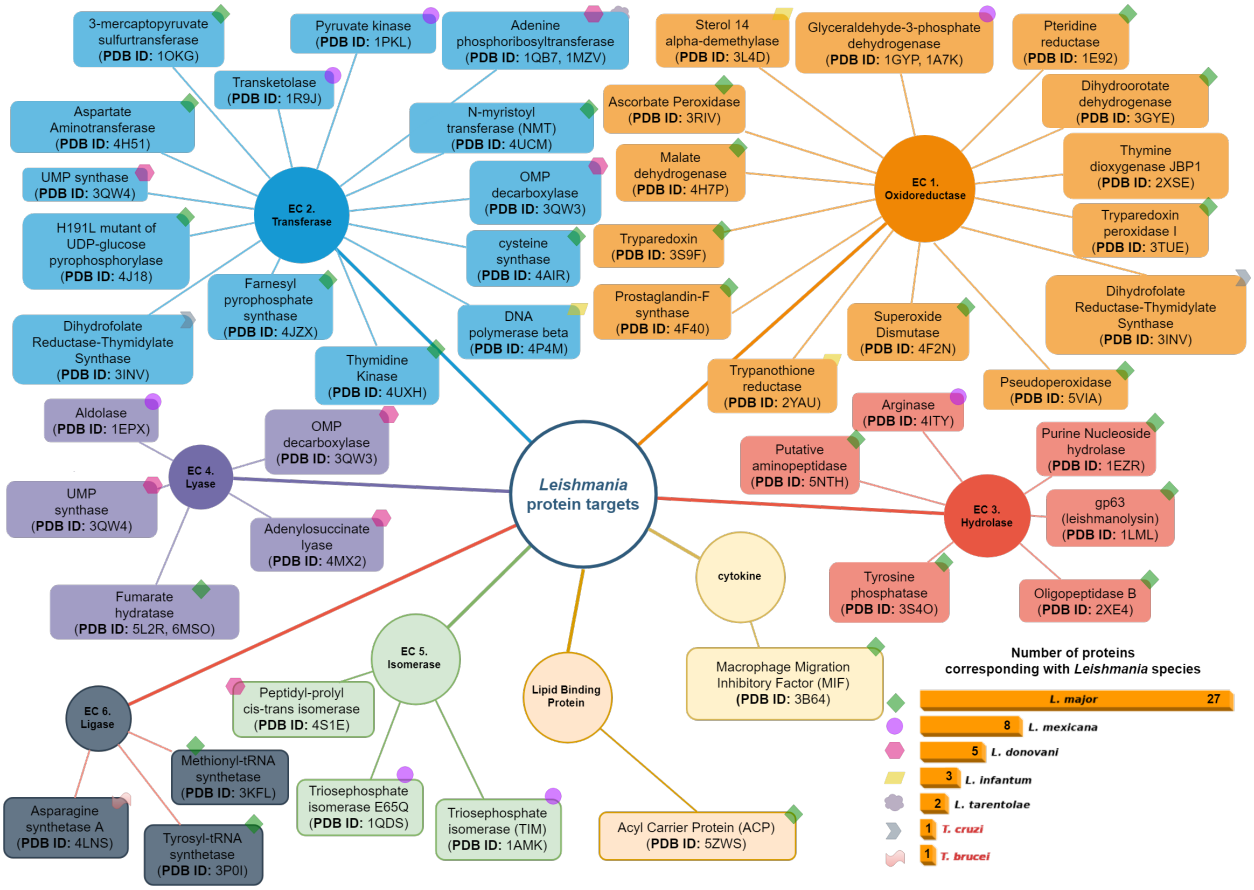

Figure 4: *Leishmania* protein classification. Oxidoreductases (orange), transferases (blue), hydrolases (red), lyases (violet), isomerases (green), ligases (dark blue), cytokines (yellow) and Lipid Binding protein (light orange). The chart at the bottom right of the figure shows the number of proteins 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.[45,73]

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.[37] 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.[74] Purine nucleotide salvage by *Leishmania* is important process to carried parasite viability and growth, due to these parasites are

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strict purine nucleotide auxotrophs.[75,76] Therefore, these parasites have protein arsenal and some molecular mechanism to purine nucleotide acquisition.[76,77] Adenine phosphoribosyltransferase (APRT) (PDB ID: 1QB7 and 1MZV) belongs to the phosphoribosyltransferase family type I (PRTs)[78] and is involved in purine-salvaging process, catalyzing adenines to adenosine-5-monophosphate (AMP) compound.[76,77] The two most common forms of protein fatty acylation are modification with myristate molecule and other.[79] Modifications with myristate, know as myristoylation, have been implicated in targeting protein to membrane locations, stabilizing protein structures, mediating protein-protein interactions and substrate activation.[79,80] Proteins that are destined to become myristoylated begin its primary sequence with the Methionine-Glycine (Met-Gly) sequence group, where Met- amino acid is removed by methionine amino-peptidase protein and myristate molecule is linked via an amide bond.[79] N-myristoyltransferase (NMT) protein (PDB ID: 4UCM) catalyzes the co-translational transfer of myristic acid (myristate) from myristoyl-CoA to the N-terminal glycine.[80,81] 4UCM is important protein by *Leishmania* parasites, and this protein is a potential drug target.[82]

The *de novo* pyrimidine biosynthesis pathway involve six enzymatic steps carried to the synthesis of Uridine 5'-monophosphate (UMP), where, the final two enzymatic steps are mediated by Orotate Phosphoribosyltransferase (OPRT) and Orotidine 5'-monophosphate decarboxylase (OMPDC) enzymes.[48,83] These two enzyme are expressed as a bifunctional protein, know as UMP synthase.[48] PDB-database have bifunctional UMP synthase protein (PDB ID: 3QW4) and OMP decarboxylase single protein (PDB ID: 3QW3) crystal structures. Trypanothione compound play an important role in maintaining intracelullar redox homoeostasis and providing defence against oxidative stress in *Leishmania* parasites.[84–86] The Trypanothione biosynthesis process, indirectly depend on the availability of cysteine and cysteine *de novo* biosynthesis pathway depend of Serine Acetyltyltransferase and Cysteine Synthase (PDB ID: 4AIR) proteins.[84,87] Amastigote *Leishmania* parasites survive and proliferate within phagolysosome vacuole, under extreme acid environment conditions and

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several toxic compounds.[88] These extreme host cell conditions cause high levels of DNA damage to parasite.[89] Therefore, DNA Polymerase Beta (PDB ID: 4P4M) is essential to *Leishmania* parasites for maintenance, replication and recombination of DNA.[89] This protein is specially required to amastigote parasite forms.[89]

Process to obtain pyrimidine and purine nucleotides in *Leishmania* parasites, may be through *de novo* biosynthesis and/or salvage pathways[90] *Leishmania* species have both pyrimidine *de novo* biosynthesis and salvage pathways, but are incapable of synthesising purines *de novo*.[90] Thymidine kinase (TK) (PDB ID: 4UXH) is an essential enzyme that initialize the pyrimidine salvage process.[91] This protein 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.[92] Farnesyl pyrophosphate synthase (FPPS) (PDB ID: 4JZX) is involved in ergosterol synthesis, acting in the early steps of isoprene synthesis and maintainance of lipid bilayer integrity.[93,94] It is a potential enzymatic target, because, it was successful inhibited with bisphosphonate previously.[93] UDP-glucose pyrophosphorylase *L. major* (UGP) (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+}, as part of the glycolytic pathway.[74]

Aspartate aminotransferase (AAT) (PDB ID: 4H51) catalyzes the 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).[95] It is pyridoxal phosphate (PLP) cofactor dependent and a potential drug target.[96] Sulfurtransferases are a family enzyme widely distributed on prokaryotes and eukaryotes organism.[97] 3-mercaptopyruvate sulfurtransferase (PDB ID: 1OKG) belong to this family and is involved in cysteine metabolism, polarizing the carboxyl group of 3-mercaptopyruvate through a tiophilic attack.[98] Transketolase (PDB ID: 1R9J) is a key enzyme to the nonoxidative branch of the PP pathway, which transfers two-carbon glycolaldehyde units from ketose-donors to aldose-acceptor sugars.[99] Finally, pyruvate kinase (PDB ID: 1PKL) catalyzes the phosphoenolpyruvate-phosphate group

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transfer to adenosine diphosphate. This enzyme is involved in the glycolytic pathway.[100]

Hydrolases group (EC. 3)

Hydrolases catalyze reactions in which a bond in any substrate is hydrolyzed to produce two fragments.[37] Due to the *Leishmania* parasite are incapable to make *de novo* biosynthesis process of purines, Purine Nucleoside Hydrolase *L. major* enzyme (PDB ID: 1EZR) is the main responsible of nucleotide salvaging from the host.[101] 1EZR is a potential drug target. The *Leishmania* parasite promastigote expresses glycoproteins on its surface, and one of these enzymes expressed is known as Leishmanolysin (*gp63* gene) (PDB ID: 1LML).[102] 1LML protein play an important role in the macrophage infection process, therefore, this enzyme is a potential drug target.[102] Peptidase proteins family play key roles in metabolic pathways, host invasion and parasite immune evasion to most parasites.[103] Oligopeptidase B (OPB) *L. major* (PDB ID: 2XE4) protein belong to peptidase family, and some studies citated by McLuskey,[104] say that 2XE4 protein is an important virulence factor.[104,105] Another peptidase enzyme is Leucyl aminopeptidase (LAP) *L. major* (PDB ID: 5NTH), which is involved in N-terminus catalysis of proteins.[106]

Tyrosine phosphatase (PRL-1) (PDB ID: 3S4O) is mainly secreted by the *Leishmania* parasite promastigote form, however, the amastigote produces PRL-1 more efficiently and abundantly specifically during the macrophage infection process,[107] which is important for the parasite survival. Finally, the arginase protein (PDB ID: 4ITY) catalyzes the first step of polyamine biosynthesis. This process makes part of the cellular growth process, and its of paramount importance for parasite survival.[108]

Lyase group (EC. 4)

Lyase enzymes are a group of enzymes that catalyzes non-hydrolytic reactions, in which a chemical group is cleaved and removed from any substrate, leaving a double bond.[37] Adenylosuccinate lyase (ASL) (PDB ID: 4MX2) is a lyase protein, and have been identified as

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vital component of purine salvaging in *Leishmania donovani*.[76] Fumarate hydrolase (FH) class 1 enzyme is a protein with two isoforms: a mitochondrial (PDB ID: 6MSO) and a cytosolic ((PDB ID: 5L2R)). 5L2R produces fumarate substrate for the dihydroorotate dehydrogenase. Additionally, this enzyme migrates to the cellular nucleus, playing a key rol in DNA repair processes.[109] 6MSO catalyzes the stereospecific reversible conversion of fumarate to S-malate. This reaction is part of the tricarboxylic acid (TCA) cycle, takes part of the succinic fermentation pathway, participates in DNA repair processes and is proposed to provide fumarate for the *de novo* pyrimidine biosynthetic pathway.[110] Finally, aldolase *L. mexicana* (PDB ID: 1EPX) enzyme, is involved in the glycolytic pathway and catalyzes the Fructose-1,6-bisphosphate conversion to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate.[111]

Isomerases group (EC. 5)

Isomerase enzymes catalyze one-substrate/one-product reactions that can be regarded as isomerization reactions.[37] Triosephosphate isomerase (TIM) (PDB ID: 1AMK) plays a preponderant role in the glycolysis process as catalyst of dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde-3-phosphate (GAP).[112] A TIM E65Q mutant (PDB ID: 1QDS) has been studied and is regarded as more stable than the TIM wild-type.[113] 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`.[114]

Ligases group (EC. 6)

Ligase enzymes catalyze bond formation between two or more macromolecules, it process usually are associated with hydrolysis of a small chemical molecule coupled to the macromolecules.[37] For this review, ligase group has associated three proteins recognized as potential enzymatic targets: Methionyl-tRNA synthetase (PDB ID: 3KFL)and Tyrosyl-tRNA

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synthetase (PDB ID: 3P0I), with structural parameters elucidated with MgATP as substrate and methionine as solvent and recognized as essential for biological processes such as gene translation.[115,116] The third 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, 4NLS protein was recognized as causing of growth delay in parasite and it was catalogued as a potential drug target for *Leishmania* bioactive principles development.[73]

Cytokines group

The Migration Inhibitory Factor (MIF) from *L. major* (PDB ID: 3B64) has been also recognized as a possible drug development target. This cytokine is an ortholog of human MIF, also known as Lm1740MIF. 3B64 interacts with MIF receptors, such as 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.[117]

Lipid Binding Protein group

Apo- Acyl Carrier Protein (PDB ID: 5ZWS) plays an important role in the synthesis of fatty acids, non-ribosomal polypeptides and polyketides. The fatty acid pathway, and their more complex forms, recently gained attention in *Leishmania* research studies, because it plays a role in protozoan parasites survival inside the host.[118]

Proteomics analysis: *Leishmania* parasite metabolic pathways

Leishmania proteins obtained in this review are involved in critical and important metabolic pathways, *e.g.* glycolysis, PPP, citric acid cycle. These proteins and pathways are essential

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for metabolic host infection and evasion processes, therefore, these are critical for *Leishmania* parasite survival.[119] Some proteins participate in several pathways performing one or more functions, *e.g.* Arginase participates in arginine biosynthesis, proline and arginine metabolism, secondary metabolite and antibiotic biosynthesis pathways.[120]

We have made a deep review of scholar literature databases and structural, chemical and physical information sources, creating a large *Leishmania* metabolic pathway's compilation (see fig. 5). Proteins with PDB ID 1E92, 2XSE, 3VIA, 4F2N, 4F40, 4UCM, 2XE4, 3S4O, 5NTH, 4S1E, 3P0I, 3KFL y 5ZWS do not appear in the metabolic databases reviewed, and other proteins as 3B64, 1LML and 4P4M, are structural proteins or play different roles into *Leishmania* parasite, because are not included into any specific metabolic pathway in figure 5. In the Figure 6 contain all proteins obtained and filtrated in this review, showing all pathway where these are associated. Proteins not associated to any path, are schematized too, but with the follow description: "Not included in pathway". Protein EC functions are discriminated with colors, therefore, protein with two or more colors have two or more functions.

Leishmaniasis drugs

Pentavalent antimonials (Sb(V)) were the first developed *Leishmania* control bioactive chemicals, however, development of *Leishmania* resistance rendered the Pentavalent Antimmonials highly inefficient. New drugs and treatments research are towards the development[2] of more effective drugs, because standard drugs generates secondaty effects as high toxicity, also, these treatments and drugs are expensibe and generates resistance for *Leishmania* parasites.[121,122] These reasons mentioned above make a leishmaniasis diseases one type of Neglected Tropical Disease (NTD). The NTD does not have necessaries economic funds and strict epidemiological controls from governmental organizations, made difficult drugs and treatments development.[123]

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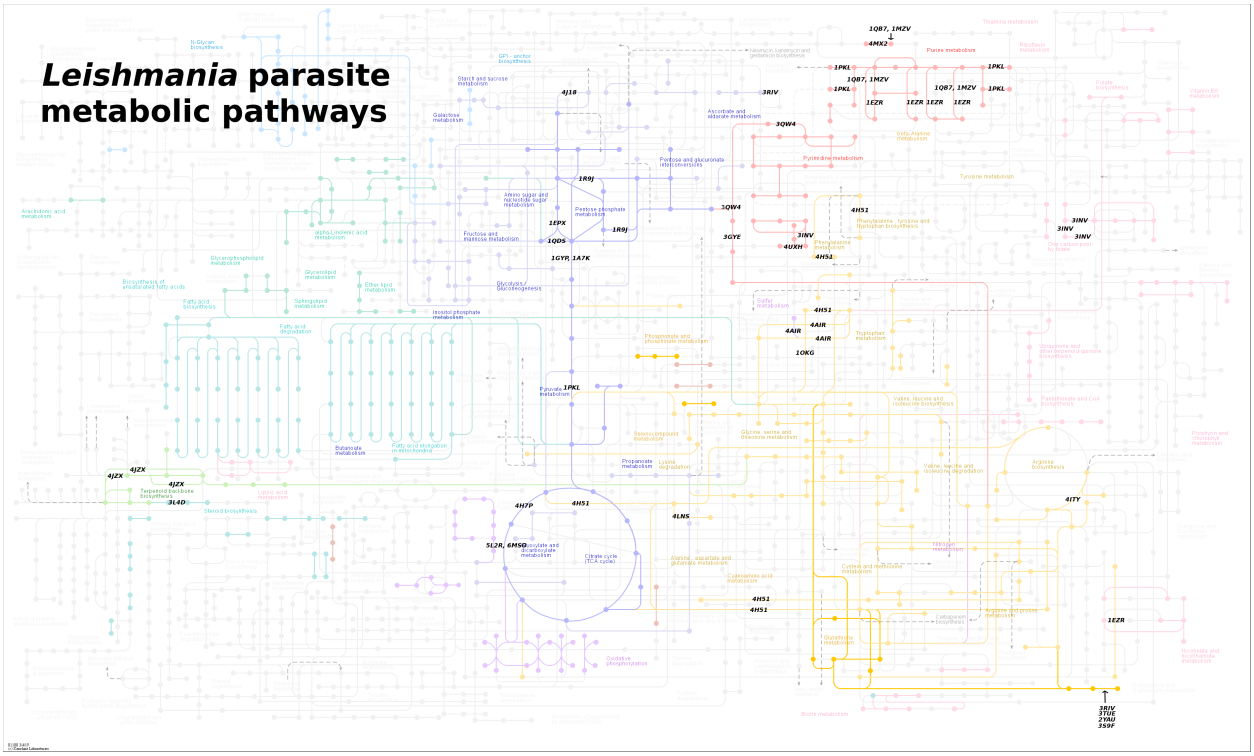

Figure 5: This figure show *Leishmania* metabolic pathways related with all proteins compiled in this paper. Proteins PDB ID 1E92, 2XSE, 3VIA, 4F2N, 4F40, 4UCM, 2XE4, 3S4O, 5NTH, 4S1E, 3P0I, 3KFL y 5ZWS, do not appear in the metabolic pathways databases reviewed

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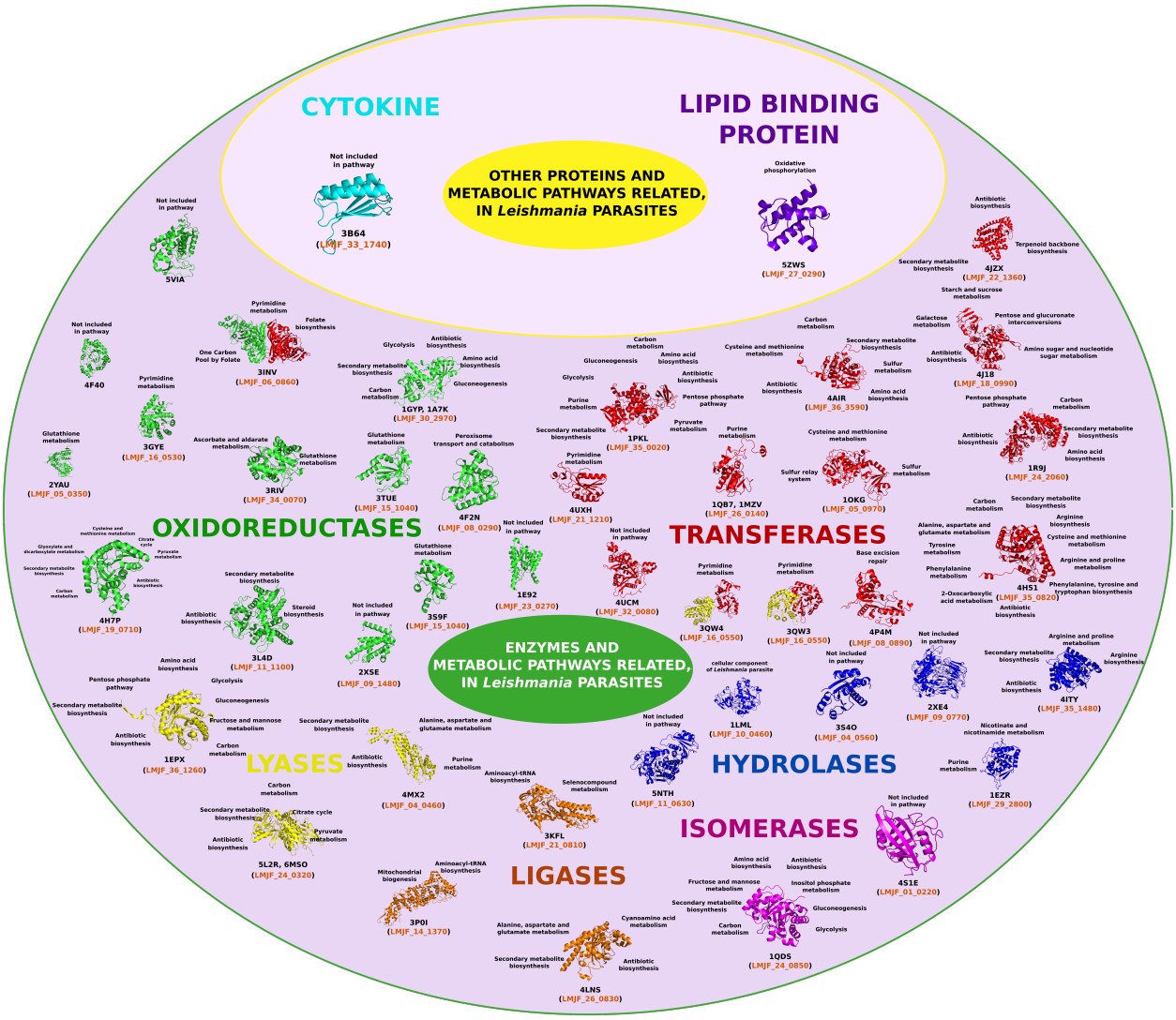

Figure 6: This figure shows proteins related with different *Leishmania* metabolic pathways. This figure include proteins without metabolic pathway defined on literature reviewed

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Standard drugs

Currently, leishmaniasis treatment are based on the following types of chemicals: antimonials (Sb(V)), amphotericin B, Pentamidine, Miltefosine (hexadecylphosphocholine), paromomycin (aminosidine), sitamaquine and pamidronate (see Fig. 7).[124,125] These drugs are used for the treatment of either cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) or visceral leishmaniasis (VL).[121]

Antimonials were the first antileishmania compounds, introduced in the 40s decade.[2,124] They are available as meglumine antimoniate (Glucantime) and sodium stibogluconate (Pentostam). These are standard first line drugs for treatment, but emergence of resistance has limited their use.[122,126] Antimonials are used for VL treatment, but, different studies found that *L. donovani* and *L. braziliensis* are more sensitive to sodium stibogluconate than *L. major*, *Leishmania tropica* and *L. mexicana*.[2,125] Amphotericin B is a macrolide antibiotic isolated from *Streptomyces nodosus* in 1956 and widely used since the 80s as amphotericin B deoxycholate.[2,124] It selectively inhibits the membrane synthesis of the parasite and causes holes in the membrane, leading to parasite death.[124] 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. 7).[124,125] Amphotericin B and its lipid formulations are used as alternative chemotherapeutic treatments.[126] Lipid formulations of amphotericin B have gained more importance, becoming the established leishmaniasis treatment by the US Food and Drug Administration (FDA).[2]

Pentamidine antileishmania activity centers on the 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. Also, monetary costs renders them prohibitive.[124]

India and East Africa used paromomycin as a cheap alternative treatment, despite its

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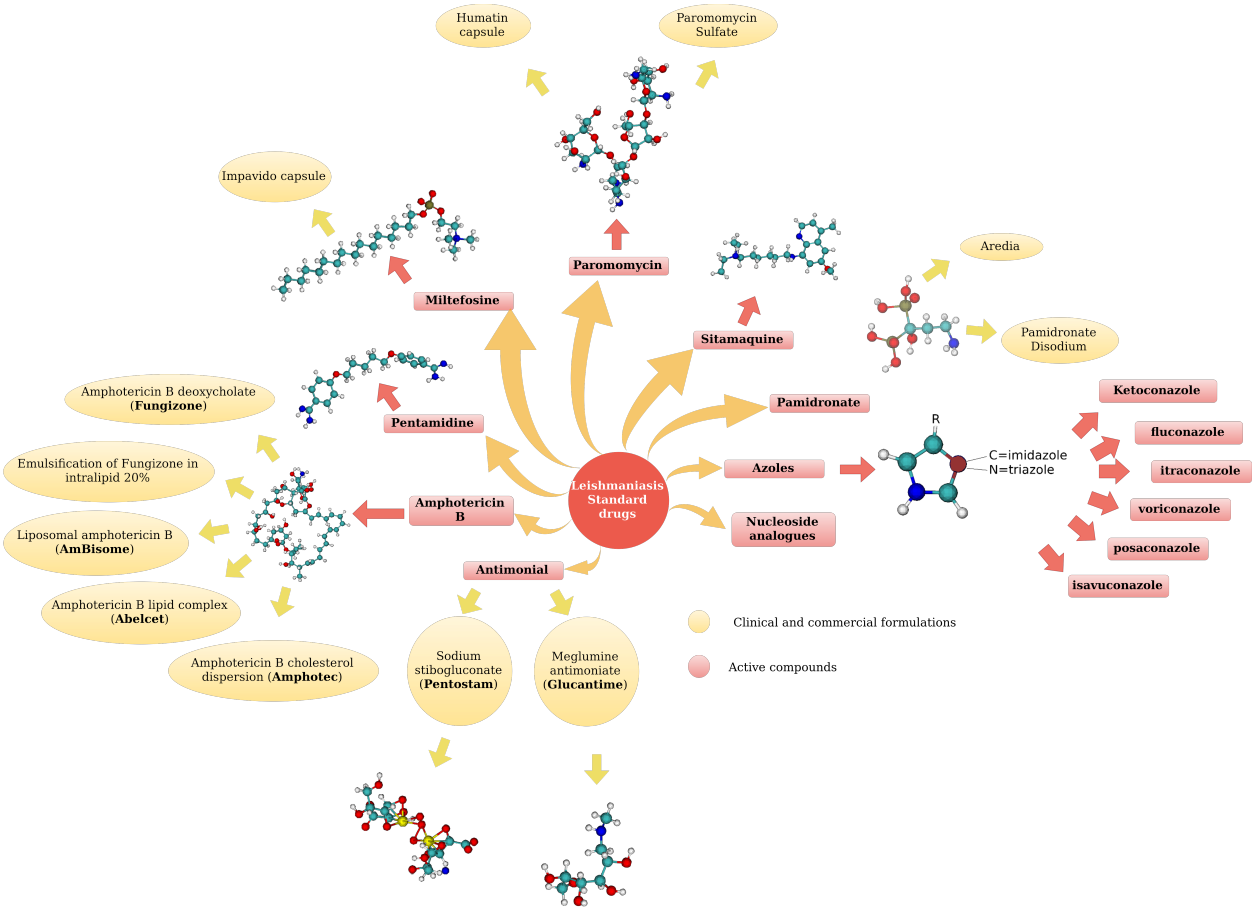

Figure 7: Drugs used against leishmanias can be divided in two main groups: standard drugs and alternative drugs (metabolites). Graphics show molecular structures of drug active compounds. Structures were obtained from ChemSpider (http://www.chemspider.com/).

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toxicity.[2] Paromomycin remained neglected until the 80s, when topical formulations for VL were developed.[124] 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.[124]

Miltefosine, initially developed as an anticancer drug, currently is the first effective oral treatment of VL.[2,124] 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.[125] 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.[124,127] 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.[124] Other two type of drugs considered for leishmaniasis treatment are azoles and nucleoside analogues.[125,128]

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.[125]

Metabolomics: Non standard drugs from plants

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 reduced duration of therapy, eventually leading to reduced secondary toxic effects of the drugs.[121] Some natural compounds, such as secondary plant metabolites, animal compounds and another type of alternative treatments.

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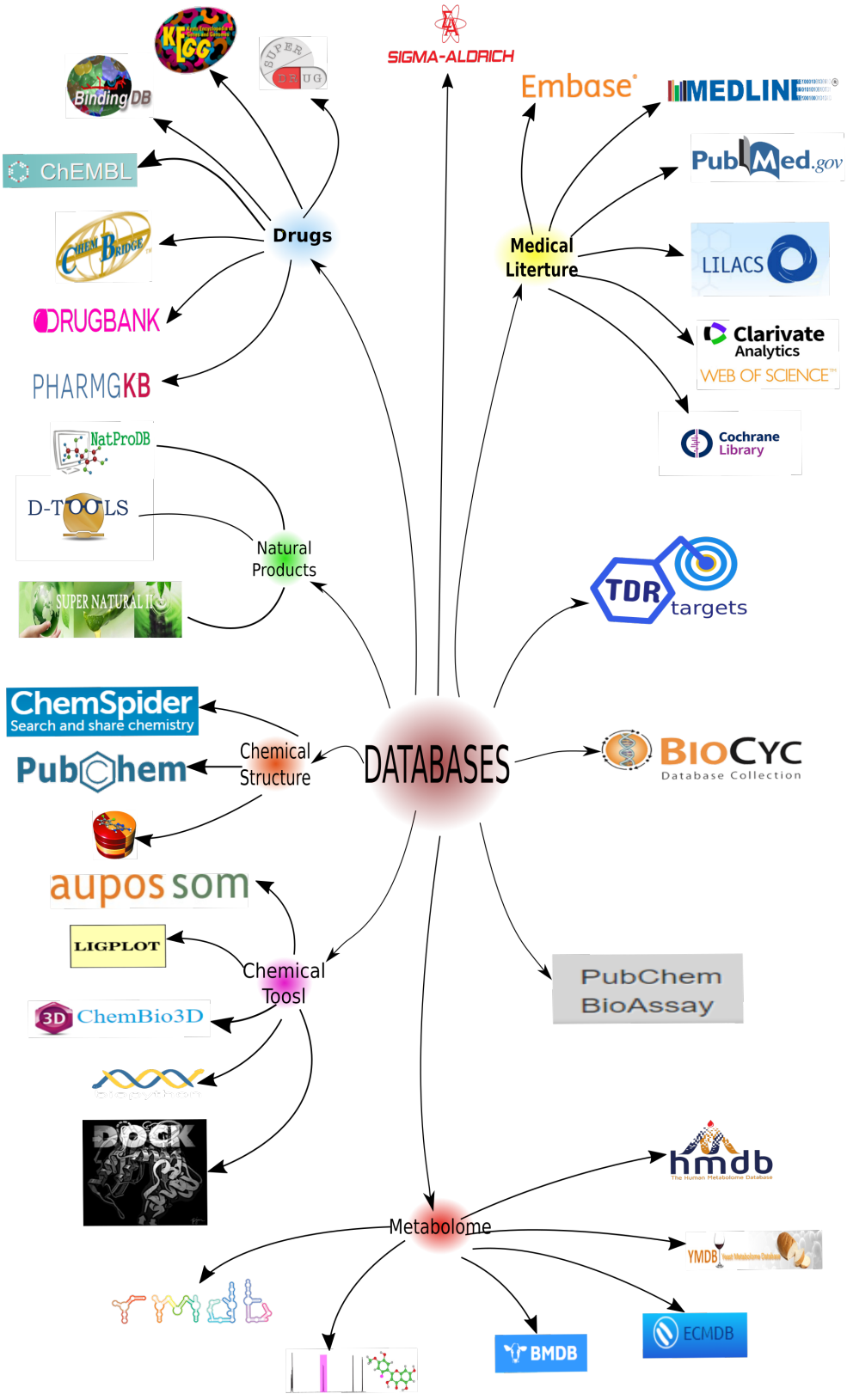

Figure 8: Drug-database review. This figure show a wide drug-database, some database exclusivity related with antileishmanial drugs

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Previous studies and reviews show approximately 200 compounds isolated from plants recognized as antileishmanicides.[129–131] These compounds were extracted from different parts of the plant anatomy and tested in experimental (*in vitro* and *in vivo*) assays under different physical chemistry conditions and different *Leishmania* species. Recently, antimicrobial peptides (AMPs) have been a major type of compounds used as antibiotics. These are produced by all kinds of living organisms and acting on viruses, bacteria, fungi and parasites.[132,133] In this research, drug databases reviewed are compiled in the figure 8, and are discriminated between metabolome, chemical-tools, chemical-structure, natural-products and medical-literature databases.

Acknowledge

Authors acknowledge Universidad Nacional de San Agustn (UNSA, Arequipa, Per) for providing access to INKARI (High Performance Computer Facilities, located at the Astronomic Observatory of CHARACATO - UNSA, Arequipa, Per). Also, we acknowledge Dr. Wilson M. Castro Silupu for building the scientific cooperation bridges UNSA-UNISUCRE, and M.Sc. Wilder Nina C. for technological support with INKARI.

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