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Article

The Future of Leishmaniasis Treatment: Protein Targets and Beyond

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Abstract: This review summarizes up-to-date research on Leishmania protein structures, extracted primarily from the Protein Data Bank (PDB), that are involved in metabolic pathways of the parasite causing *Leishmania*. Additionally, we examine current advancements in identifying and developing bioactive chemical agents to treat this largely neglected tropical disease. We examined experimental data from *in vitro*, *in vivo*, or *in silico* sources, classifying the information into four categories: a) vector taxonomy and geographical distribution; b) parasite taxonomy and geographical distribution; c) enzymatic functions of proteins involved in parasite/host interactions across developmental stages (e.g. oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases, and cytokines); and d) standard and experimental treatments using bioactive chemical compounds. Our goal is to provide a reference framework for research focused on elucidating interaction mechanisms and ligand-protein activation/inactivation processes related specifically to *Leishmania* infections. We therefore highlight enzymes known to participate in biochemical pathways triggered during Leishmania infection episodes. This review summarizes current knowledge to inform and guide future discovery efforts targeting proteins and pathways for improved *Leishmania* disease management.

Keywords: Leishmania; Trypanosoma; Molecular Dynamics; Theoretical Chemistry

1. Overview

Leishmaniasis is a tropical and subtropical group of neglected zoonotic diseases, caused by species of the *Leishmania* genus [1? ?], which mainly affects mammals and is transmitted through the bite of infected female sandflies [2–6].

The World Health Organization (WHO) categorizes leishmaniasis as a Neglected Tropical Disease (NTD) due to the limited effective treatments and inadequate investment in research and development [7–11]. As a result, global cases of the predominant forms - cutaneous (CL) and visceral (VL) leishmaniasis - continue to rise annually. Though mucocutaneous (MCL) leishmaniasis also occurs, WHO surveillance focuses on the most widespread CL and VL forms. CL is the most common clinical manifestation worldwide [12,13]. CL leishmaniasis is the most common form (see Figure 1), while VL more severely impacts internal organs like the spleen and liver, making it more dangerous and potentially fatal.

In 2021, Brazil reported the highest number of cases globally for both cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL), with 16,800 cases of CL and 1,492 cases of VL. Colombia recorded the second highest number 6,175 cases of CL and 7 cases of VL. These combined reports accounted for 71% of all leishmaniasis cases in the New World [14]. However, when comparing the prevalence of the disease, Colombia had a higher prevalence (0.012%) compared to Brazil's (0.0075%). In terms of incidence, Brazil reported 6.94 cases of CL per 100,000 people, while Colombia recorded 12.10 cases per 100,000 people. Based on the latest report from the World Health Organization (WHO) on the culmination of the past decade (2021), these data emphasize the ongoing significance of leishmaniasis as a public health issue in both countries.

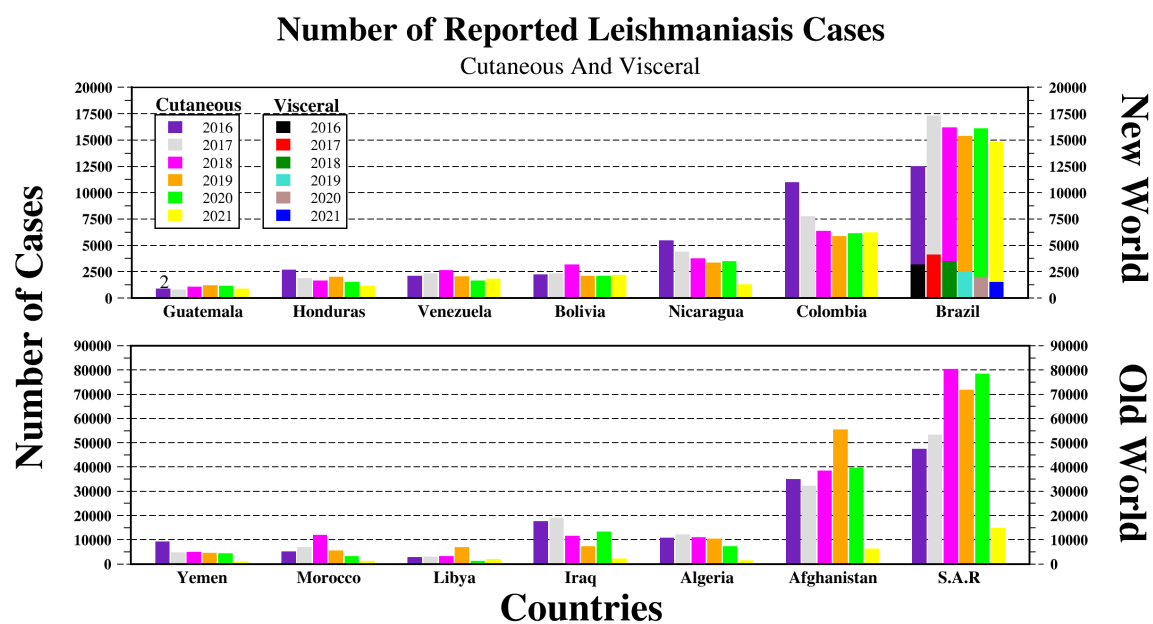


Figure 1. Number of Visceral and Cutaneous Leishmaniasis cases reported by the World Health Organization (WHO). The figure shows the most relevant reports in the world (new and old world).

Leishmania species are taxonomically grouped and classified into two main divisions called "sections": Euleishmania and Paraleishmania [15,16]. These divisions were proposed by Cupolillo et al. (2000) based on molecular and phylogenetic studies of *Leishmania* species, using the genus *Endotrypanum* as an external phylogenetic group to construct a dendrogram. The phylogenetic results demonstrated evolutionary divergences among several established *Leishmania* species (Nei's genetic distance (D) = 1.04). Cupolillo utilized various molecular techniques including Multilocus Enzyme Electrophoresis (MLEE), Restriction Fragment Length Polymorphism (RFLP) analysis of the intergenic transcribed spacers (ITSrRNA) in the rRNA gene cluster, measurement of sialidase activity, and primary DNA sequencing of the small subunit (SSU) rRNA gene to compare and verify their results [15,17,18].

According to Cupolillo and colleagues, additional species have been included in the two proposed divisions. Currently, Euleishmania encompasses the following subgenera: *L. (Viannia)*, *L. (Leishmania)*, and *L. (Sauroleishmania)* (Figure 3) [15,19–21]. On the other hand, Paraleishmania includes the subgenus *L. (Endotrypanum)*, which consists of the species *E. schaudinni* and *E. monterogeii* (See Figure 3) [22–25]. Specifically in Colombia, reports indicate the presence of *L. amazonensis*, *L. braziliensis*, *L. mexicana*, *L. colombiensis*, *L. guyanensis*, *L. panamensis*, *L. chagasi*, *L. lainsoni*, and *L. equatoriensis* as transmission parasites of leishmaniasis [26–29].

Currently, standard active compounds used against *Leishmania* parasites include Amphotericin B, Miltefosine, Pentamidine, Antimonials, Paromomycin, Sitamaquine, Pamidronate, Azoles, and

Nucleoside analogues.[30–36], However, these drugs often demonstrate inefficiency and high toxicity in patients undergoing clinical treatments. The effectiveness and toxicity of these drugs depend on various factors, such as the immunological state of the infected host and the pharmacokinetic properties of the drugs themselves. For example, Miltefosine-based drugs are teratogenic, and antimonial treatments can lead to adverse effects like arthralgia, nausea, abdominal pain, pancreatitis, and cardiotoxicity [37–39]. Experimental development and efficacy testing of potential antileishmanial compounds have proven to be challenging and costly, with a low success rate. The development of new drugs is undoubtedly a complex endeavor. As an alternative, computational approaches such as Molecular Dynamics and Molecular Docking offer a more suitable avenue for studying potential antileishmanial compounds and protein targets. These approaches enable the analysis of numerous ligand-protein interactions with relatively low computational cost, providing a detailed atomistic/molecular description compared to experimental assays.

The development of novel drugs derived from alternative chemical sources, such as secondary metabolites and peptides extracted from plants and animal species, holds great potential for the advancement of more efficient and effective treatments compared to conventional approaches.

The objective of this study is to review the state-of-the-art protein targets for the *Leishmania* parasite. We conducted a systematic search of several databases, including the Protein Data Bank (PDB), books, and articles. We also review alternative compounds, such as those derived from small secondary metabolites and proteins.

2. *Leishmania* Vectors

Sandflies play a crucial role as vectors for various dangerous parasites, including *Leishmania*, *Bartonella bacilliformis* bacteria, and certain viruses, which can have severe consequences, even leading to the death of the host [40–44]. These tiny insects belong to the Diptera order, Psychodidae family, and Phlebotominae subfamily. Taxonomically, sandflies are classified into five genera: *Phlebotomus* and *Sergentomyia*, prevalent in the Old World, and *Lutzomyia*, *Brumptomyia*, and *Warileya*, prevalent in the New World [45–47]. Among these genera, *Phlebotomus* and *Lutzomyia* are particularly responsible for the transmission of *Leishmania* parasites [45,48] (See Figure 2).

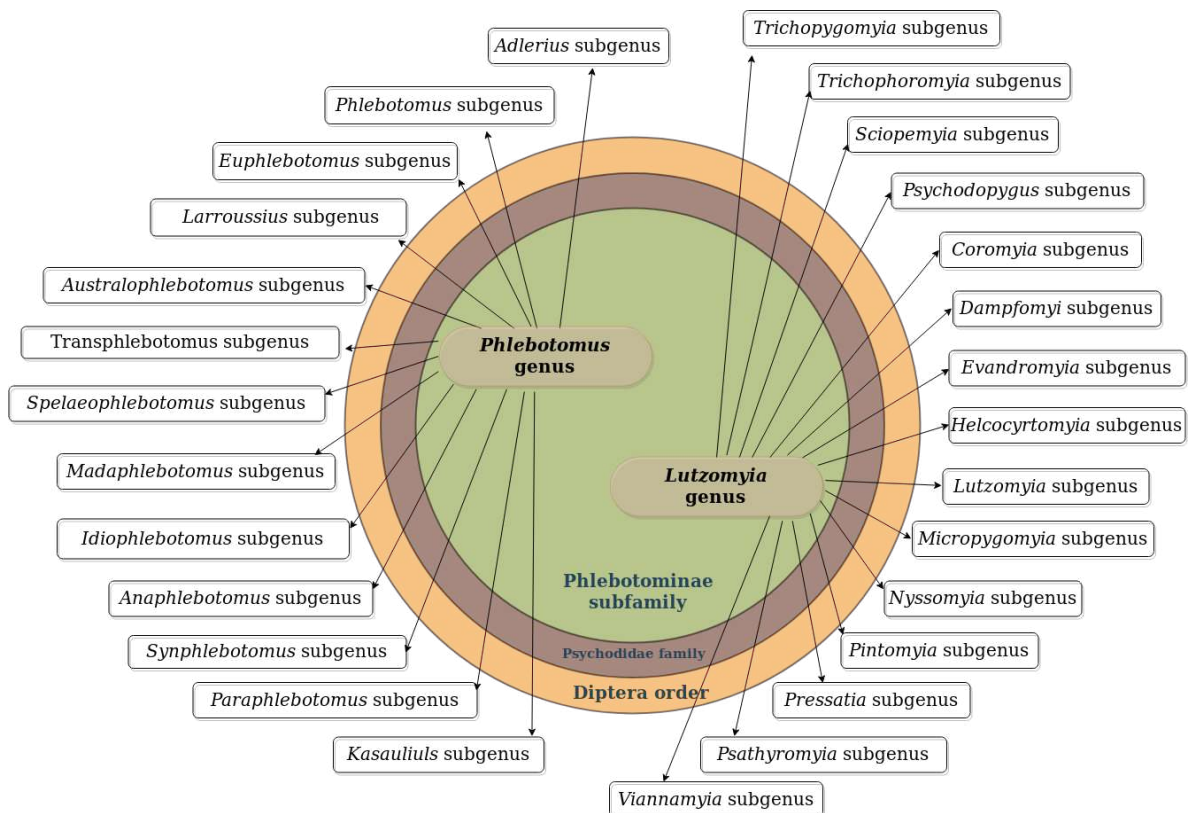
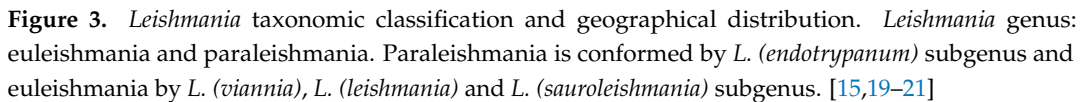


Figure 2. Taxonomic distribution of *Leishmania* vector: Diptera order and Psychodidae family, phlebotominae subfamily. Geographical distribution: *Phlebotomus* genus for the old world and *Lutzomyia* genus for the new world. [17,23]

Nine species of the *Leishmania* genus and fourteen species belonging to the *Lutzomyia* genus have been reported for Colombia. [14,27] Parasite species reported for Colombia are *L. amazonensis*, *L. braziliensis*, *L. mexicana*, *L. colombiensis*, *L. guyanensis*, *L. panamensis*, *L. infantum*, *L. lainsoni* and *L. equatoriensis* (see Figure 3). *Lutzomyia* species Colombia reported are *L. flaviscutellata*, *L. colombiana*, *L. spinicrassa*, *L. pia*, *L. towsendi*, *L. hartmanni*, *L. umbratilis*, *L. longifloca*, *L. trapidoi*, *L. panamensis*, *L. yuli yuli*, *L. cruciata*, *L. columbiana* and *L. gomezi* [14,49,50].



Leishmania parasites invade, develop, and replicate inside the host Mononuclear Phagocyte System (MPS), targeting macrophages and dendritic cells [51–54]. The incubation period for the transformation of *Leishmania* promastigotes to amastigotes ranges from two to three months, during which the host's immune system response determines the outcome, whether favorable or unfavorable [52]. The parasite cycle initiates with the bite of an infected female sandfly carrying promastigote *Leishmania* parasites in a metacyclic state (see Figure 4) [55–57]. At this stage, neutrophils and macrophages are the first line of defense activated by the immune system, with neutrophils initiating the inflammatory response [54,56,58].

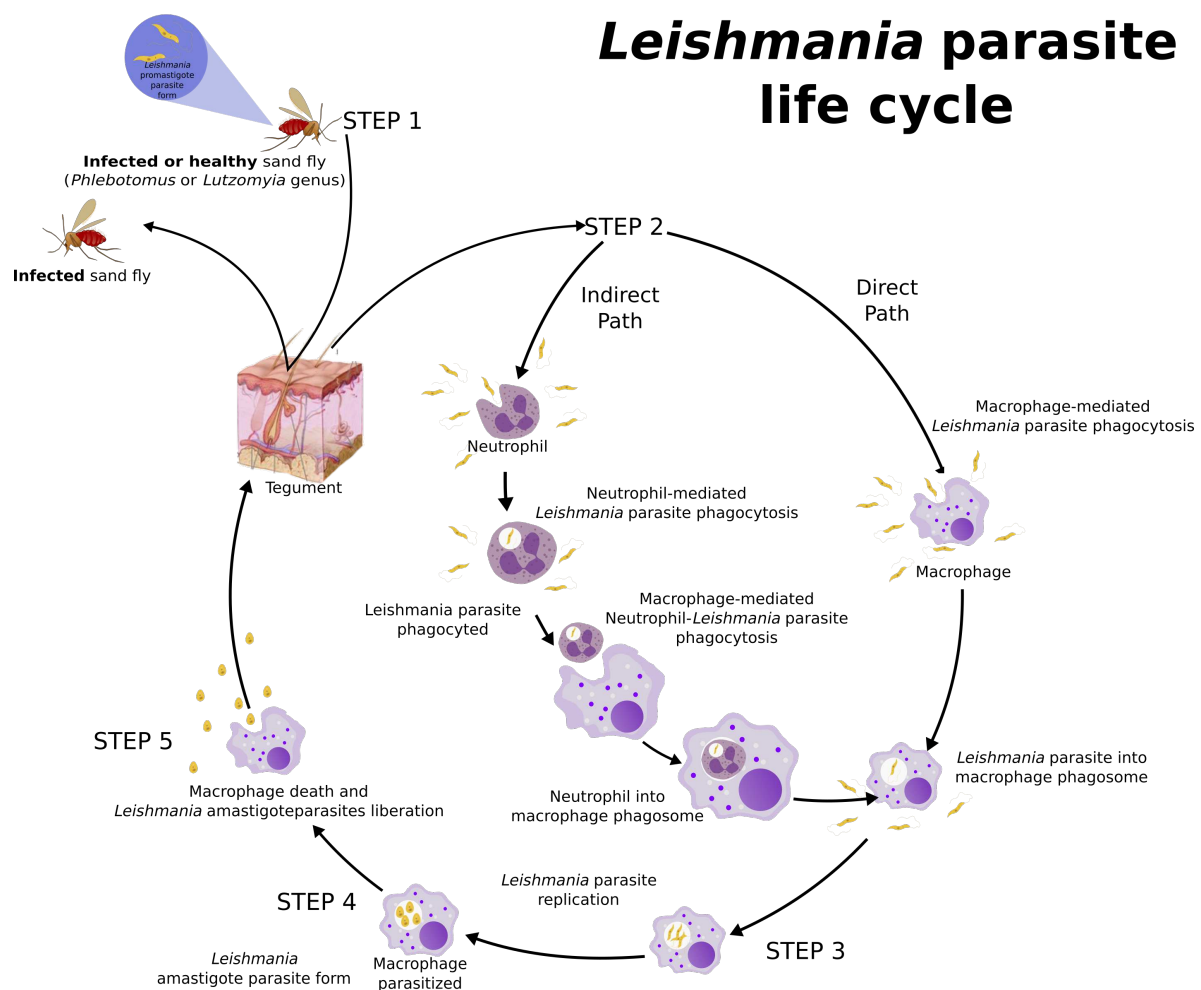


Figure 4. *Leishmania* life cycle can be divided in five steps. **Step 1:** Virulent metacyclic promastigotes are egested when a female sandfly carrier bites a possible host. **Step 2:** Here there are two possible paths. Direct path: promastigotes are phagocytized by macrophage cells. Indirect path: 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.

The *Leishmania* parasite employs two mechanisms to enter macrophages: a direct pathway involving the macrophage itself and an indirect pathway through the attack on neutrophils [59]. In the direct pathway, promastigotes are endocytosed by macrophages and enclosed within a phagosome, which subsequently undergoes biochemical transformation into a phagolysosome [60]. The indirect pathway involves the phagocytosis of the parasite by neutrophils, followed by subsequent phagocytosis by macrophages (see Figure 4) [59].

Within the phagolysosome vacuole, the promastigote parasite state survives by producing specific factors such as Lipophosphoglycan (LPG), gp63 protein, and glutathione transferase [55,61].

Following the initial attack on macrophages, the host immune system responds by triggering a biochemical cascade that involves the production of cytokines by specialized CD4⁺ T-helper (T_H) cells, specifically T_H1 or T_H2 cells. Among the cytokines secreted by T_H1 cells, gamma interferon (IFN-γ) plays a crucial role. IFN-γ activates and stimulates macrophages, enhancing their antimicrobial

activity. Additionally, interleukin (IL)-12 and IL-2, which are also secreted by T_H1 cells, promote the differentiation of $CD4^+$ T cells into the T_H1 phenotype. This phenotypic transformation is important for an effective response against the exogenous attack, in this case, the metacyclic promastigote form of *Leishmania* [62,63].

The immune T_H2 response produces IL-4, IL-5, IL-10, the Transforming Growth Factor Beta (TGF- β) and other cytokines. These compound are perfectly suited for facing allergenic diseases or helminthic infections, 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.[62,64]

4. Leishmania Proteomics and Metabolomics Analysis

We conducted an extensive review of databases and scientific literature on *Leishmania* metabolic pathways. Based on the gathered information, we analyzed, organized, and correlated the pathways and proteins involved, as depicted in Figure 5. The proteins illustrated in Figure 5 are essential components of vital metabolic pathways such as glycolysis, PPP, citric acid cycle, among others. They play crucial roles in host infection and immune evasion processes, thereby being critical for the survival of *Leishmania* [65]. Some proteins have multiple functions across different metabolic pathways. For example, Arginase participates in arginine biosynthesis, proline and arginine metabolism, as well as secondary metabolite and antibiotic biosynthesis pathways [66]. However, proteins 1E92, 2XSE, 3VIA, 4F2N, 4F40, 4UCM, 2XE4, 3S4O, 5NTH, 4S1E, 3P0I, 3KFL, and 5ZWS were not found in the reviewed metabolic databases and were therefore not assigned to specific pathways in Figure 5. Nonetheless, we thoroughly reviewed and analyzed the functions of these proteins in this paper.

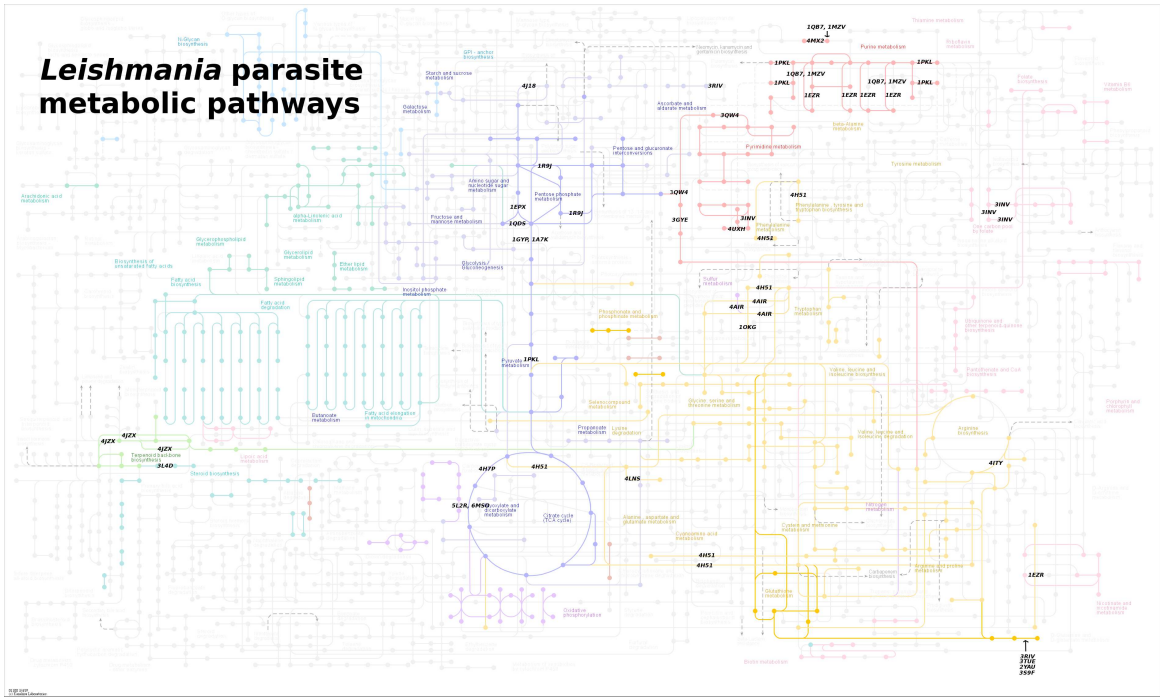
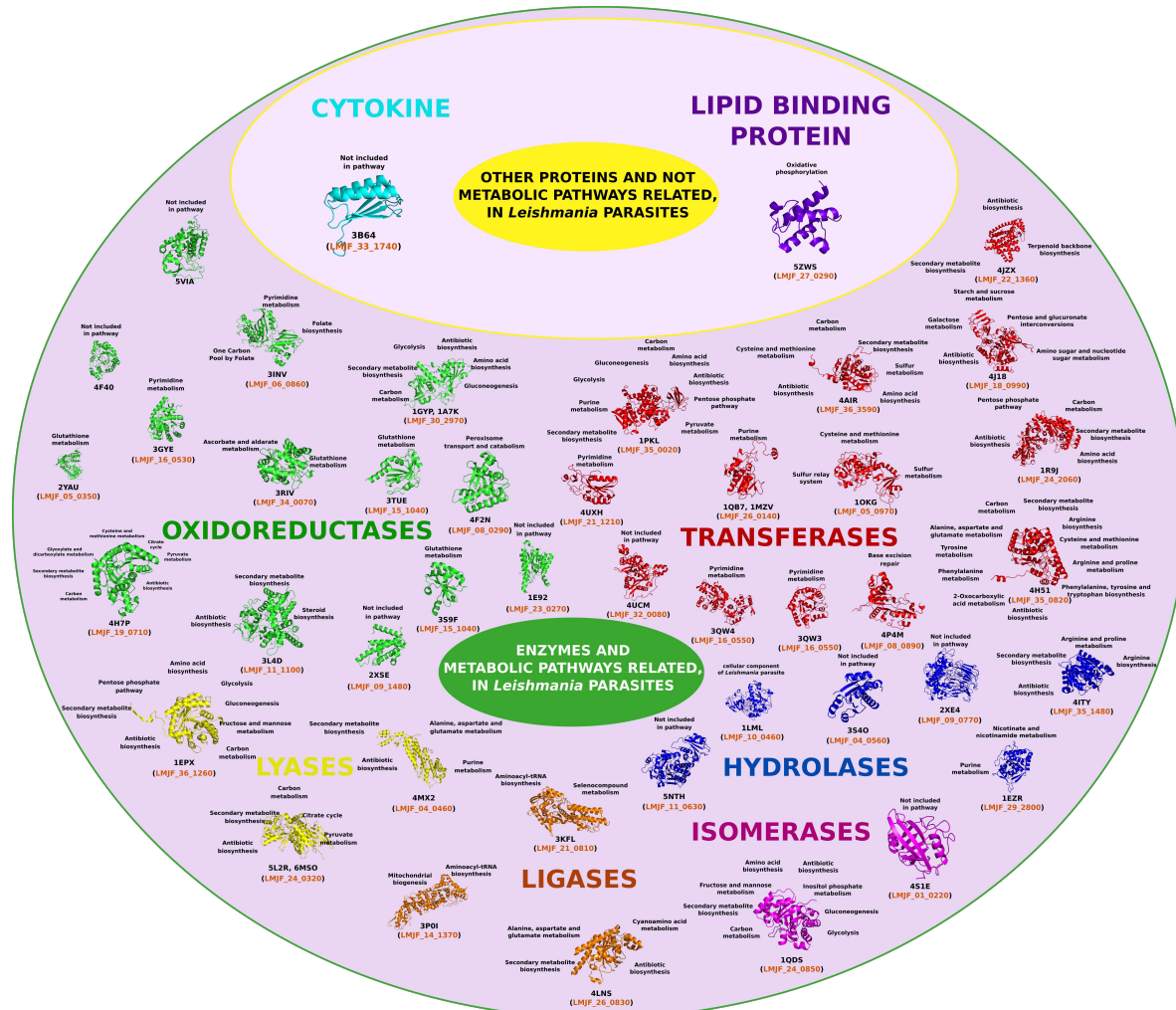


Figure 5. This figure shows *Leishmania* metabolic pathways related with retrieved proteins from databases. The PDB IDs 1E92, 2XSE, 3VIA, 4F2N, 4F40, 4UCM, 2XE4, 3S4O, 5NTH, 4S1E, 3P0I, 3KFL and 5ZWS, do not appear in metabolic pathway databases

Lastly, proteins 3B64, 1LML, and 4P4M are structural macromolecules that play various roles in the *Leishmania* parasite. They do not belong to any specific metabolic pathway and are primarily considered for structural analysis. It is worth emphasizing the significance of these proteins in the context of *Leishmania* and their potential implications for further research in understanding the parasite's metabolism and pathogenicity. Figure 6 shows protein-pathway correlations as well, highlighting



The crystal structures presented in this study were obtained from the Protein Data Bank (PDB) [67]. A search was conducted in the PDB using the keywords "Leishmaniasis" and "Leishmania," resulting in a total of 700 items available until 2023. The selected proteins were then classified based on their relevance to the parasitization cycle. To ensure diversity in the protein selection, other criteria were considered. For instance, proteins with different PDB codes but identical structures were included, as well as proteins with the same structure but elucidated from different organisms. An example of such proteins is the Dihydrofolate Reductase-Thymidylate Synthase (DHFR-TS) (PDB ID: 3INV), which functions as both an oxidoreductase and a transferase.

Ultimately, the study group comprised a total of 49 proteins, as shown in Figure ???. Among these proteins, the largest groups were oxidoreductases (Enzyme Commission Number - EC 1) and transferases (EC 2), each containing 15 representatives. Additionally, there were 6 hydrolases (EC 3), 5 lyases (EC 4), 3 isomerases (EC 5), 3 ligases (EC 6), one cytokine, and one Lipid Binding Protein (Figure ???). These findings provide valuable insights into the diverse enzymatic functions and molecular characteristics of the selected proteins, shedding light on their potential roles in the biology of *Leishmania* parasites.

4.1. Oxidoreductases (EC 1)

The group of oxidoreductase enzymes identified in this study plays a crucial role in catalyzing oxidation-reduction reactions, where a substrate donates electrons to an electron-withdrawing species, resulting in the substrate being oxidized [68]. These enzymes are essential for the Glycolysis and Pentose Phosphate Pathways (PPP), which are key metabolic pathways in Trypanosomatids, including *Leishmania* parasites [69].

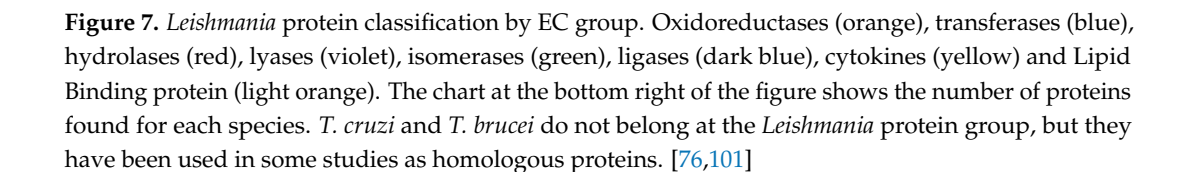
Glucose and other hexoses are vital nutrients for *Leishmania* parasites, and these parasites, whether in the promastigote or amastigote form, can acquire glucose from the extracellular environment or synthesize it through gluconeogenesis [70]. The promastigote parasites are capable of both glucose uptake and gluconeogenesis, while the amastigote parasites mainly rely on the gluconeogenic pathway for their glucose requirements [70]. These findings highlight the importance of oxidoreductase enzymes in the metabolic processes of *Leishmania* parasites, particularly in relation to glucose metabolism and energy production. Understanding the role of these enzymes and the specific pathways involved can provide valuable insights for developing targeted interventions against *Leishmania* infections.

Glyceraldehyde 3-phosphate dehydrogenase from *Leishmania (major) mexicana*, represented by PDB IDs 1GYF and 1A7K (see Figure 7), is an oxidoreductase enzyme that plays a role in both the Pentose Phosphate Pathway and glycolysis metabolic pathways [69]. This enzyme is a crucial component of glycolysis and is a homotetrameric protein with a molecular weight of 156 kDa. It catalyzes the conversion of glyceraldehyde 3-phosphate (G3P) to 1,3-bisphosphoglycerate (1,3-BPG), simultaneously reducing NAD^+ to NADH using the NAD^+ cofactor [71,72]. Another important enzyme involved in the gluconeogenic process is malate dehydrogenase, represented by PDB ID 4H7P. This enzyme participates in the conversion of oxaloacetate (OAA) to malate and vice versa, utilizing the NAD/NADH coenzyme system [73,74]. It is worth noting that 4H7P has two isoforms in eukaryotes, which differ in their subcellular localization and their specificity for the coenzymes NAD (all types of malate dehydrogenases) or NADP (only malate dehydrogenases from chloroplast cells) [74]. These enzymes, glyceraldehyde 3-phosphate dehydrogenase and malate dehydrogenase, play vital roles in *Leishmania*'s metabolic pathways, contributing to the conversion of key substrates and the production of important intermediates in glycolysis and gluconeogenesis.

Dihydrofolate Reductase-Thymidylate Synthase (DHFR-TS), represented by PDB ID 3INV, is an enzyme found in *Leishmania major*. It combines the functions of two enzymes, dihydrofolate reductase (DHFR) and thymidylate synthase (TS), in a single polypeptide chain. DHFR-TS is involved in the salvage pathway of folate compounds in trypanosomatid parasites, including *Leishmania*. These parasites are unable to synthesize folate de novo and rely on the salvage pathway to obtain folate compounds for their metabolic processes. Folate compounds are crucial for various metabolic pathways in *Leishmania*, including nucleic acid and protein biosynthesis [75,76].

Pteridine reductase (PTR1), represented by PDB ID 1E92, is an enzyme found in *Trypanosoma cruzi*, another member of the Trypanosomatidae family. PTR1 is also involved in the salvage pathway of folate compounds. It plays a crucial role in the metabolism of folate and pteridine derivatives in trypanosomatids, including *Leishmania*. The salvage of folate compounds through PTR1 is essential for the normal metabolic processes of *Leishmania* parasites, as they are auxotrophic for folate and require these compounds for nucleic acid and protein biosynthesis [75,76].

Both DHFR-TS and PTR1 are important enzymes in the salvage pathways of folate compounds in *Leishmania* parasites. Their functions are critical for the synthesis of essential biomolecules and the survival of the parasites. Understanding the structure and function of these enzymes can provide valuable insights for the development of therapeutic strategies against *Leishmania* infections. The structure of the DHFR enzyme varies significantly between host organisms and parasites, leading to the development of specific DHFR inhibitors known as antifolates. These inhibitors have been utilized as therapeutic agents against parasites, including *Leishmania* infections [77].



Eukaryotic unicellular kinetoplastid flagellates, such as *Trypanosoma* and *Leishmania* species, possess a unique hypermodified base in their nuclear DNA known as J base or β -D-glucosylhydroxymethyl-uracil [80–82]. The presence of the J base is associated with the silencing of telomeric expression sites for variant surface glycoprotein (VSG) genes, except in actively transcribed VSG genes [80,81]. The biosynthesis and maintenance of the J base in these parasites rely on the JBP1 protein, which is essential for the growth and survival of *Leishmania* parasites. JBP1 contains a DNA-binding JBP1 domain (DB-JBP1), represented by PDB ID 2XSE, that specifically binds to J-DNA. This interaction between DB-JBP1 and J-DNA makes JBP1 a potential target for drug development. Understanding the structure and function of DB-JBP1 and its role in J base biosynthesis and maintenance can provide valuable insights for the development of drugs aimed at disrupting this

process and inhibiting the growth and survival of *Leishmania* parasites [83,84]. Targeting JBP1 could offer new therapeutic strategies for combating *Leishmania* infections.

Macrophages employ various defense mechanisms against *Leishmania* parasites, including the production of toxic compounds such as peroxynitrite, hydroxyl radicals, hydrogen peroxide, hydroperoxide, superoxide radicals, and others [85–87]. These compounds can disrupt the parasite's metabolism and hinder its survival. However, *Leishmania* parasites have developed a trypanothione-mediated hydroperoxide metabolism to counteract the effects of oxidative agents, whether endogenous or exogenous [88]. Tryparedoxin (represented by PDB ID: 3S9F) and Tryparedoxin peroxidase I (represented by PDB ID: 3TUE) (TXN/TXNPx) are proteins that play a crucial role in reducing macrophage-generated hydroperoxides to water [89]. These proteins exist in a cytosolic form and are involved in the detoxification pathway, which is essential for the parasite's survival [89]. Pseudoperoxidase *L. major* (LmPP) (PDB ID: 5VIA) is a heme protein expressed by *Leishmania* parasites as a defense mechanism against Reactive Nitrogen Species (RNS) [90,91]. Heme peroxidases, such as LmPP, utilize peroxides to oxidize various physiologically important molecules. Ascorbate peroxidase (APX) (PDB ID: 3RIV) is another example of a heme peroxidase and is considered a potential drug target [90].

Understanding the structure and function of these proteins involved in the detoxification pathways can provide insights for developing novel therapeutic approaches to disrupt the parasite's defense mechanisms and enhance the efficacy of anti-*Leishmania* treatments [88–91]. 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 Reactive Oxygen Species (ROS) intermediates, such as hydrogen peroxide, OH^- , O_2^- radicals, and peroxynitrite, as part of the macrophage mechanism to fight invasive microorganisms [92–94]. Thus, 4F2N acts as the first line of defense against those ROS. This fact makes 4F2N a suitable enzymatic target for *Leishmania* drug development [92].

Trypanothione reductase (TR) (PDB ID: 2YAU) is a homodimeric enzyme extracted from *Leishmania infantum* and is essential for parasite survival [95]. This enzyme catalyzes the reduction of trypanothione by NADPH, protecting the parasite against oxidative damage and toxic heavy metals [95–97]. TR reduces trypanothione disulfide (TS_2), a bis(γ -L-glutamyl-L-cysteinylglycine) spermidine or bis(glutathionyl) spermidine conjugate, to the di-thiol form $[\text{T}(\text{SH})_2]$ [96].

The enzyme 9,11-endoperoxide prostaglandin H2 reductase from *L. major* (PDB ID: 4F40) is involved in the lipid metabolic pathway, acting through an NADP cofactor [98,99]. Sterol 14 α -demethylase (CYP51) from *L. infantum* (PDB ID: 3L4D) is an enzyme that catalyzes the removal of the 14 α -methyl group from sterol precursors. This reaction is essential for membrane cell biosynthesis, specifically, CYP51 is involved in the ergosterol pathway and is believed to be decisive for the survival of *Leishmania* parasites [100]. Understanding the structures and functions of these enzymes involved in parasite defense mechanisms, oxidative stress response, and essential metabolic pathways provides valuable insights for the design of novel therapeutic strategies against *Leishmania* infections [92–95,98,100].

4.2. Transferases Group (EC. 2)

Transferases are enzymes that catalyze reactions involving the transfer of a chemical group from an electron/proton donor substrate to an electron/proton withdrawing substrate [68]. These proteins play crucial roles in various cellular processes across all kingdoms of life, including DNA repair, RNA editing, and activation of metabolites in catabolic pathways [102]. In the case of *Leishmania* parasites, the purine nucleotide salvage process is vital for their viability and growth, as these parasites are strict purine nucleotide auxotrophs [103,104]. To acquire purine nucleotides, *Leishmania* parasites employ a protein arsenal and molecular mechanisms [104,105]. Adenine phosphoribosyltransferase (APRT) (PDB ID: 1QB7 and 1MZV) belongs to the phosphoribosyltransferase family type I (PRTs)

[106] and is involved in the purine salvage process. APRT catalyzes the conversion of adenine to adenosine-5-monophosphate (AMP) [104,105].

Protein fatty acylation is a common form of protein modification, with myristoylation being one of the most prevalent types [107]. Myristoylation plays roles in targeting proteins to membrane locations, stabilizing protein structures, mediating protein-protein interactions, and substrate activation [107,108]. Proteins destined for myristoylation typically start their primary sequence with a Methionine-Glycine (Met-Gly) dipeptide, where the initiator methionine residue is removed by methionine aminopeptidase, and a myristate molecule is linked via an amide bond [107].

N-myristoyltransferase (NMT) protein (PDB ID: 4UCM) is responsible for the co-translational transfer of myristic acid (myristate) from myristoyl-CoA to the N-terminal glycine of target proteins [108,109]. NMT plays a crucial role in *Leishmania* parasites and is considered a potential drug target [110]. Understanding the structures and functions of transferases, such as APRT and NMT, involved in purine salvage and protein modification processes, respectively, can provide insights for the development of targeted therapies against *Leishmania* infections.

The *de novo* pyrimidine biosynthesis pathway is a series of six enzymatic steps that lead to the synthesis of Uridine 5'-monophosphate (UMP). The final two steps of this pathway are mediated by the enzymes Orotate Phosphoribosyltransferase (OPRT) and Orotidine 5'-monophosphate decarboxylase (OMPDC) [111,112]. In *Leishmania* parasites, these two enzymes are expressed as a bifunctional protein known as UMP synthase [112]. Crystal structures of the bifunctional UMP synthase protein (PDB ID: 3QW4) and the OMP decarboxylase single protein (PDB ID: 3QW3) are available in the PDB database. Trypanothione plays a crucial role in maintaining intracellular redox homeostasis and providing defense against oxidative stress in *Leishmania* parasites [113–115]. The biosynthesis of trypanothione indirectly depends on the availability of cysteine, and the *de novo* biosynthesis pathway of cysteine relies on the enzymes Serine Acetyltransferase and Cysteine Synthase (PDB ID: 4AIR) [113,116].

Amastigote *Leishmania* parasites survive and proliferate within the phagolysosome vacuole, which exposes them to extreme acidic conditions and various toxic compounds [117]. These harsh host cell conditions cause high levels of DNA damage in the parasite [118]. Therefore, DNA Polymerase Beta (PDB ID: 4P4M) plays an essential role in *Leishmania* parasites for the maintenance, replication, and recombination of DNA [118]. This protein is particularly required in the amastigote form of the parasite [118]. Understanding the structures and functions of enzymes involved in pyrimidine biosynthesis, trypanothione metabolism, and DNA repair can provide insights into the development of targeted therapies against *Leishmania* infections.

In *Leishmania* parasites, both pyrimidine and purine nucleotides can be obtained through *de novo* biosynthesis and salvage pathways [119]. However, it is important to note that *Leishmania* species are incapable of synthesizing purines *de novo* [119].

Thymidine kinase (TK) (PDB ID: 4UXH) is an essential enzyme that initiates the pyrimidine salvage process in *Leishmania* parasites [120]. This enzyme catalyzes the transfer of the γ -phosphate group from ATP to 2'-deoxythymidine (dThd), forming thymidine monophosphate (dTMP). TK plays a key role in the parasitization process and is an important target for drug development [121]. Farnesyl pyrophosphate synthase (FPPS) (PDB ID: 4JZX) is involved in ergosterol synthesis and is responsible for the early steps of isoprene synthesis, as well as the maintenance of lipid bilayer integrity in *Leishmania* parasites [122,123]. FPPS is considered a potential enzymatic target, as it has been successfully inhibited with bisphosphonates [122].

UDP-glucose pyrophosphorylase *L. major* (UGP) (PDB ID: 4J18) is an enzyme involved in the glycolytic pathway of *Leishmania* parasites [102]. It catalyzes the reversible conversion of glucose-1-phosphate (Glc-1-P) and uridine 5'-triphosphate (UTP) to UDP-glucose (UDP-Glc) and inorganic pyrophosphate (PPi) in the presence of Mg^{2+} . UGP plays a role in carbohydrate metabolism and is important for the synthesis of essential glycoconjugates in the parasite [102]. Understanding the pathways involved in nucleotide biosynthesis and the enzymes associated with these processes can provide valuable insights for the development of targeted therapies against *Leishmania* infections.

Aspartate aminotransferase (AAT) (PDB ID: 4H51) is an enzyme that catalyzes the reversible transfer of the α -amino group between aspartate and glutamate [124]. Specifically, it converts L-aspartate and 2-oxoglutarate to oxaloacetate and L-glutamate. This enzymatic reaction is essential for amino acid metabolism and the synthesis of key molecules in the cell. AAT relies on the pyridoxal phosphate (PLP) cofactor, which plays a crucial role in the catalytic mechanism of the enzyme [124]. PLP acts as a coenzyme, facilitating the transfer of the amino group between the amino acid substrates. The dependence of AAT on PLP makes it an attractive target for drug development, as inhibiting its activity can disrupt amino acid metabolism and lead to detrimental effects on the parasite. Targeting AAT with specific inhibitors can interfere with the normal functioning of *Leishmania* parasites, disrupting their ability to synthesize essential molecules and causing metabolic imbalances. Consequently, AAT represents a potential drug target for the development of novel therapies against *Leishmania* infections [125].

Sulfurtransferases are a diverse family of enzymes that play important roles in sulfur metabolism in both prokaryotes and eukaryotes. One member of this family is 3-mercaptopyruvate sulfurtransferase (PDB ID: 1OKG), which is involved in cysteine metabolism. This enzyme catalyzes the transfer of a sulfur atom from 3-mercaptopyruvate to various sulfur acceptor molecules, polarizing the carboxyl group of 3-mercaptopyruvate through a nucleophilic attack [126,127]. Transketolase (PDB ID: 1R9J) is another important enzyme involved in carbohydrate metabolism. It plays a key role in the nonoxidative branch of the pentose phosphate pathway (PP pathway). Transketolase transfers two-carbon glycolaldehyde units from ketose donors to aldose acceptor sugars, facilitating the interconversion of different sugar phosphates [128]. Pyruvate kinase (PDB ID: 1PKL) is an enzyme involved in the glycolytic pathway, specifically in the final step of glycolysis. It catalyzes the transfer of a phosphate group from phosphoenolpyruvate to adenosine diphosphate (ADP), generating pyruvate and ATP. This reaction is crucial for energy production in cells [129].

These enzymes, sulfurtransferase, transketolase, and pyruvate kinase, have important roles in various metabolic pathways and are essential for the proper functioning of cells. Understanding their structures and functions provides insights into their mechanisms of action and their potential as targets for drug development and therapeutic interventions.

4.3. Hydrolases Group (EC. 3)

Hydrolases are a class of enzymes that catalyze hydrolysis reactions, where a bond in a substrate molecule is cleaved by the addition of a water molecule, resulting in the formation of two product fragments [68]. In the case of the *Leishmania* parasite, which cannot synthesize purines de novo, the Purine Nucleoside Hydrolase of *L. major* (PDB ID: 1EZR) plays a crucial role in salvaging nucleotides from the host [130]. This enzyme is responsible for hydrolyzing purine nucleosides to release the purine base and ribose or deoxyribose [130]. Due to its essential role in nucleotide metabolism, Purine Nucleoside Hydrolase is considered a potential drug target for *Leishmania* [130].

Leishmanolysin (gp63 gene) is a glycoprotein expressed on the surface of *Leishmania* promastigotes, and its structure is represented by PDB ID: 1LML [131]. This enzyme plays a critical role in the infection of macrophages by *Leishmania* parasites, mediating the degradation of host extracellular matrix proteins and evading the immune response [131]. Consequently, Leishmanolysin is considered a potential drug target for combating *Leishmania* infections [131]. Peptidases, a family of enzymes, have diverse functions in metabolic pathways, host invasion, and immune evasion by parasites [132]. Oligopeptidase B (OPB) of *L. major* (PDB ID: 2XE4) is a peptidase enzyme known to be an important virulence factor [133,134]. It plays a role in the degradation of host proteins and the processing of parasite virulence factors [133,134].

Leucyl aminopeptidase (LAP) of *L. major* (PDB ID: 5NTH) is another peptidase enzyme involved in the catalysis of N-terminal residues of proteins [135]. It participates in the hydrolysis of peptide bonds, contributing to the processing and turnover of proteins in *Leishmania* [135]. These hydrolase enzymes, including Purine Nucleoside Hydrolase, Leishmanolysin, Oligopeptidase B, and Leucyl

aminopeptidase, have significant roles in the metabolism, virulence, and survival of *Leishmania* parasites. Understanding their structures and functions can aid in the development of drugs and interventions targeted against *Leishmania* infections.

Tyrosine phosphatase PRL-1 (PDB ID: 3S4O) is primarily secreted by the promastigote form of *Leishmania* parasites. However, during the macrophage infection process, the amastigote form produces PRL-1 more efficiently and abundantly, which is crucial for the parasite's survival [136]. This enzyme is involved in the dephosphorylation of tyrosine residues in protein substrates, regulating various signaling pathways and cellular processes [136]. The increased production of PRL-1 by amastigotes suggests its importance in the adaptation and virulence of *Leishmania* during the intracellular infection of macrophages [136].

Arginase (PDB ID: 4ITY) is an enzyme that catalyzes the hydrolysis of arginine to urea and ornithine. It plays a critical role in the first step of polyamine biosynthesis, which is essential for cellular growth and proliferation [137]. Polyamines are involved in various biological processes, including DNA synthesis, cell division, and differentiation [137]. In *Leishmania*, arginase is crucial for the production of polyamines, which are required for the parasite's survival and virulence [137]. Both tyrosine phosphatase PRL-1 and arginase are important enzymes for the survival and adaptation of *Leishmania* parasites. They contribute to key cellular processes and are potential targets for the development of interventions against *Leishmania* infections.

4.4. Lyase Group (EC. 4)

Adenylosuccinate lyase (ASL) (PDB ID: 4MX2) is a lyase enzyme that plays a vital role in purine salvage in *Leishmania donovani* [104]. It catalyzes the cleavage of adenylosuccinate into fumarate and AMP, participating in the recycling of purine nucleotides in the parasite [104]. Fumarate hydrolase (FH) is a lyase enzyme with two isoforms: a mitochondrial form (PDB ID: 6MSO) and a cytosolic form (PDB ID: 5L2R) [138]. The cytosolic isoform (5L2R) produces fumarate, which serves as a substrate for dihydroorotate dehydrogenase and participates in the *de novo* pyrimidine biosynthetic pathway [138]. Additionally, FH migrates to the cellular nucleus and plays a role in DNA repair processes [138].

Aldolase *L. mexicana* (PDB ID: 1EPX) is an enzyme involved in the glycolytic pathway [139]. It catalyzes the conversion of fructose-1,6-bisphosphate into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, playing a crucial role in carbohydrate metabolism [139].

4.5. Isomerases Group (EC. 5)

Isomerase enzymes catalyze the conversion of one substrate to its isomeric form, and Triosephosphate isomerase (TIM) is a notable example of such an enzyme. TIM (PDB ID: 1AMK) is primarily involved in the glycolysis process and is responsible for catalyzing the reversible isomerization between dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde-3-phosphate (GAP) [140]. This enzymatic interconversion between DHAP and GAP is a crucial step in glycolysis, as it facilitates the transformation of DHAP, a ketose, into GAP, an aldose. The resulting GAP can then proceed through subsequent metabolic reactions to produce ATP and other essential intermediates. Studies have also focused on a mutant form of TIM known as TIM E65Q (PDB ID: 1QDS), which has shown increased stability compared to the wild-type TIM [141]. This mutant variant exhibits altered kinetic properties and enhanced resistance to unfolding, providing valuable insights into the structural and functional characteristics of TIM and its stability.

Peptidyl-prolyl cis-trans isomerase (PPIase) is an isomerase enzyme involved in protein folding processes [142]. One example is the PDB entry 4S1E, which catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides [142]. PPIases play a crucial role in accelerating protein folding, aiding in the correct formation of peptide bonds and facilitating the proper conformation of proteins.

4.6. Ligases Group (EC. 6)

Ligase enzymes catalyze the formation of bonds between macromolecules through a process often associated with the hydrolysis of a small chemical molecule coupled to the macromolecules [68]. In this review, three proteins belonging to the ligase group have been identified as potential enzymatic targets: Methionyl-tRNA synthetase (PDB ID: 3KFL) and Tyrosyl-tRNA synthetase (PDB ID: 3P0I), whose structural parameters have been elucidated with MgATP as a substrate and methionine as a solvent, and are recognized as essential for biological processes such as gene translation [143,144]. The third enzyme is Asparagine synthetase A (ASNA) (PDB ID: 4LNS), an ammonium and glutamine-dependent enzyme. Experimental studies (*in vivo* and *in vitro*) have shown that the 4LNS protein causes growth delay in the parasite and has been identified as a potential drug target for the development of bioactive compounds against *Leishmania* [101].

4.7. Cytokines Group

The Migration Inhibitory Factor (MIF) from *L. major* (PDB ID: 3B64) has been identified as a potential target for drug development. This cytokine, known as Lm1740MIF, shares orthology with human MIF. 3B64 interacts with MIF receptors, such as HLA class II histocompatibility antigen gamma chain (also known as invariant chain or CD74), and exhibits antiapoptotic activity. This activity may facilitate the intracellular persistence of *Leishmania* within macrophages [145].

4.8. Lipid Binding Protein Group

The Apo-Acyl Carrier Protein (PDB ID: 5ZWS) plays a crucial role in the synthesis of fatty acids, non-ribosomal polypeptides, and polyketides. The fatty acid pathway, including its more complex forms, has recently gained attention in research on *Leishmania* due to its involvement in the survival of protozoan parasites inside the host [146].

5. Leishmaniasis Drugs

Pentavalent antimonials (Sb(V)) were the initial bioactive chemical species developed for the control of *Leishmania*. However, the emergence of *Leishmania* resistance has significantly reduced the efficacy of Pentavalent Antimonials [17]. As a result, there has been a growing focus on the development of new antileishmanial compounds. Currently, standard drugs used for treatment, such as Amphotericin B, Pentamidine, Miltefosine, Paromomycin, Sitamaquine, etc., are also facing challenges of inefficiency and high costs, leading to the development of drug resistance in *Leishmania* parasites [12,17,39].

5.1. Standard Drugs

Pentavalent antimonials (Sb(V)) were the initial bioactive chemical species developed for the control of *Leishmania*. However, the emergence of resistance in *Leishmania* parasites has significantly reduced the efficacy of pentavalent antimonials [17]. Consequently, there has been a growing emphasis on the development of new antileishmanial compounds. Currently, standard drugs used for treatment, such as Amphotericin B, Pentamidine, Miltefosine, Paromomycin, Sitamaquine, etc., are also facing challenges of inefficiency and high costs, which further contribute to the development of drug resistance in *Leishmania* parasites [12,17,39].

Antimonials, including meglumine antimoniate (Glucantime) and sodium stibogluconate (Pentostam), were the first antileishmanial compounds introduced in the 1940s [17,38]. These drugs have been the standard first-line treatment for leishmaniasis. However, the emergence of resistance has significantly limited their effectiveness [39,147]. Studies have shown that *L. donovani* and *L. braziliensis* are more sensitive to sodium stibogluconate compared to *L. major*, *Leishmania tropica*, and *L. mexicana* [17,37].

Amphotericin B, a macrolide antibiotic isolated from *Streptomyces nodosus* in 1956, has been widely used since the 1980s, particularly as amphotericin B deoxycholate [17,38]. It selectively inhibits membrane synthesis in the parasite and creates pores in the membrane, leading to parasite death [38]. Amphotericin B is commonly used as a second-line treatment and is available in various formulations, including amphotericin B deoxycholate (Fungizone), amphotericin B in intralipid 20% (Amphotericin B Intralipid), liposomal amphotericin B (AmBisome), amphotericin B lipid complex (ABLC; Abelcet), and amphotericin B cholesterol dispersion (ABCD; Amphotec) [37,38]. Lipid formulations of amphotericin B, in particular, have gained significant importance and have been established as the preferred treatment for leishmaniasis by the US Food and Drug Administration (FDA) [17].

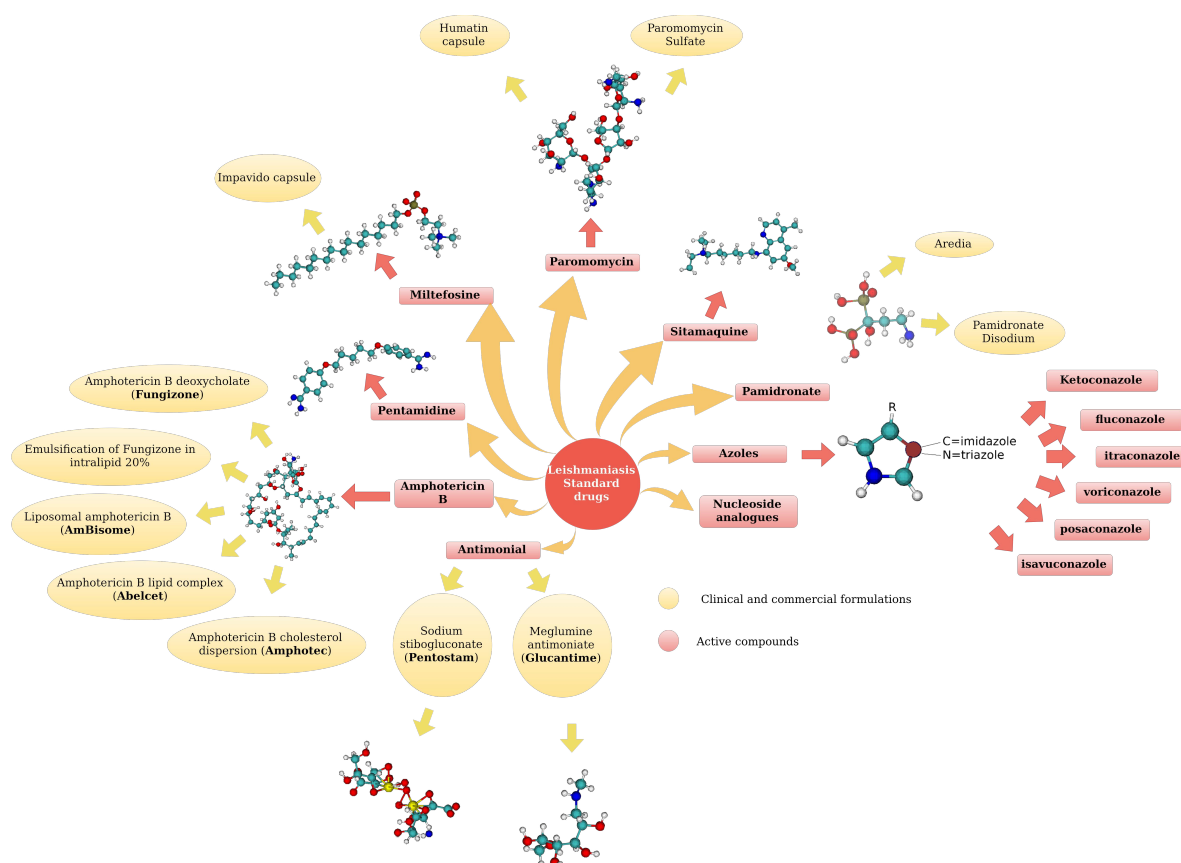


Figure 8. Drugs used against *Leishmania* can be divided in two main groups: standard drugs and alternative drugs. Graphic shows molecular structures of drug active compounds. Structures were obtained from ChemSpider database (<http://www.chemspider.com/>).

Pentamidine is an antileishmanial drug that targets the parasite's polyamine biosynthesis and mitochondrial membrane. It is considered a second-line treatment for visceral leishmaniasis (VL) due to its toxicity, which includes adverse effects such as myalgia, nausea, headache, hypoglycemia, irreversible insulin-dependent diabetes mellitus, and even death. Additionally, its high cost makes it economically prohibitive [38].

In India and East Africa, paromomycin has been used as a cheaper alternative treatment option for VL, despite its toxicity [17]. Initially, paromomycin was neglected, but in the 1980s, topical formulations for VL were developed, renewing interest in its therapeutic potential [38]. To address the issue of accessibility and affordability, organizations such as One World Health, the Bill and Melinda Gates Foundation, Gland Pharma Limited, IDA Solutions, and WHO/TDR have partnered to develop paromomycin as a public health tool to be sold on a not-for-profit basis at a significantly reduced price [38].

Miltefosine, originally developed as an anticancer drug, has emerged as the first effective oral treatment for visceral leishmaniasis (VL) [17,38]. Its efficacy has been investigated against various species of *Leishmania*, including *L. donovani*, *L. major*, *L. tropica*, *Leishmania aethiopica*, *L. mexicana*, and *L. panamensis*, in both the promastigote and amastigote stages. In vitro studies have shown varying sensitivities, with *L. donovani* being recognized as the most sensitive species to miltefosine [37]. The effective concentration (ED₅₀) of miltefosine against *L. donovani* ranges from 0.12 to 1.32 μ M [37].

Sitamaquine is another drug used for leishmaniasis treatment. It undergoes rapid metabolism, forming desethyl and 4-CH₂OH derivatives, which may contribute to its activity. Sitamaquine is relatively well-tolerated, with mild methemoglobinemia reported as a potential side effect [38,148]. Pamidronate, a bisphosphonate drug, has shown significant activity against *Leishmania donovani* in vitro. It targets the FPPS protein (PDB ID: 4JZX) and potently inhibits it in the trypanosomatid parasite [38]. Azoles and nucleoside analogues are two other types of drugs considered for the treatment of leishmaniasis [37,149]. Within the azole group, drugs such as ketoconazole and itraconazole are commonly used. They exert their antileishmanial activity by inhibiting the enzyme C14 α -demethylase, which is involved in the synthesis of ergosterol, an essential component of the *Leishmania* cell membrane [37].

Nucleoside analogues, such as allopurinol and pyrazolopyrimidine, exert their antileishmanial effects by disrupting the enzymatic processes involved in the purine salvaging pathway of *Leishmania* [37]. This pathway is essential for the parasite's ability to synthesize and utilize purine nucleotides, which are crucial for its growth and survival. By inhibiting key enzymes in this pathway, nucleoside analogues interfere with the parasite's ability to obtain the necessary building blocks for nucleic acid synthesis and energy metabolism, ultimately leading to its demise.

5.2. Metabolomics: Non standard drugs

We have compiled a list of databases containing bioactive compounds that have been investigated for their potential in treating Leishmaniasis, serving as alternatives to commercial drugs (see Figure 9) (See supplementary material). These databases cover various aspects, including metabolome analysis, chemical tools, chemical structure information, natural products, and relevant literature in the medical field. Furthermore, we have conducted an extensive review of research papers that have compiled over 200 chemical compounds and several plant extracts (See supplementary material). These studies involve a wide range of experimental techniques, both *in vivo* and *in vitro*, and encompass different *Leishmania* species, considering various physical and chemical conditions [150–152].

On the other hand, antimicrobial peptides (AMPs) have emerged as potential treatments for Leishmaniasis, particularly in *in vitro* assays. AMPs are a diverse group of compounds that are naturally produced by various organisms, including humans, and exhibit antimicrobial activity against viruses, bacteria, fungi, and parasites. In animals, AMPs are primarily found in tissues and organs and serve as a crucial component of the innate immune system's first line of defense [153,154]. Their broad-spectrum antimicrobial properties make them attractive candidates for developing novel therapeutic strategies against *Leishmania* parasites.

Is important to highlight that *Leishmania* parasites can develop resistance against these secondary metabolites and AMPs too, therefore, the use of monotherapies is not an good option. The rationale behind combined therapies is to prevent the risk of parasite resistance, to provide increased efficacy resulting from synergistic effects, minimizing dose requirements and to reduce therapy time, eventually leading to a reduction of side toxic effects, or al least to mild toxicity and reduce mortality.[12]

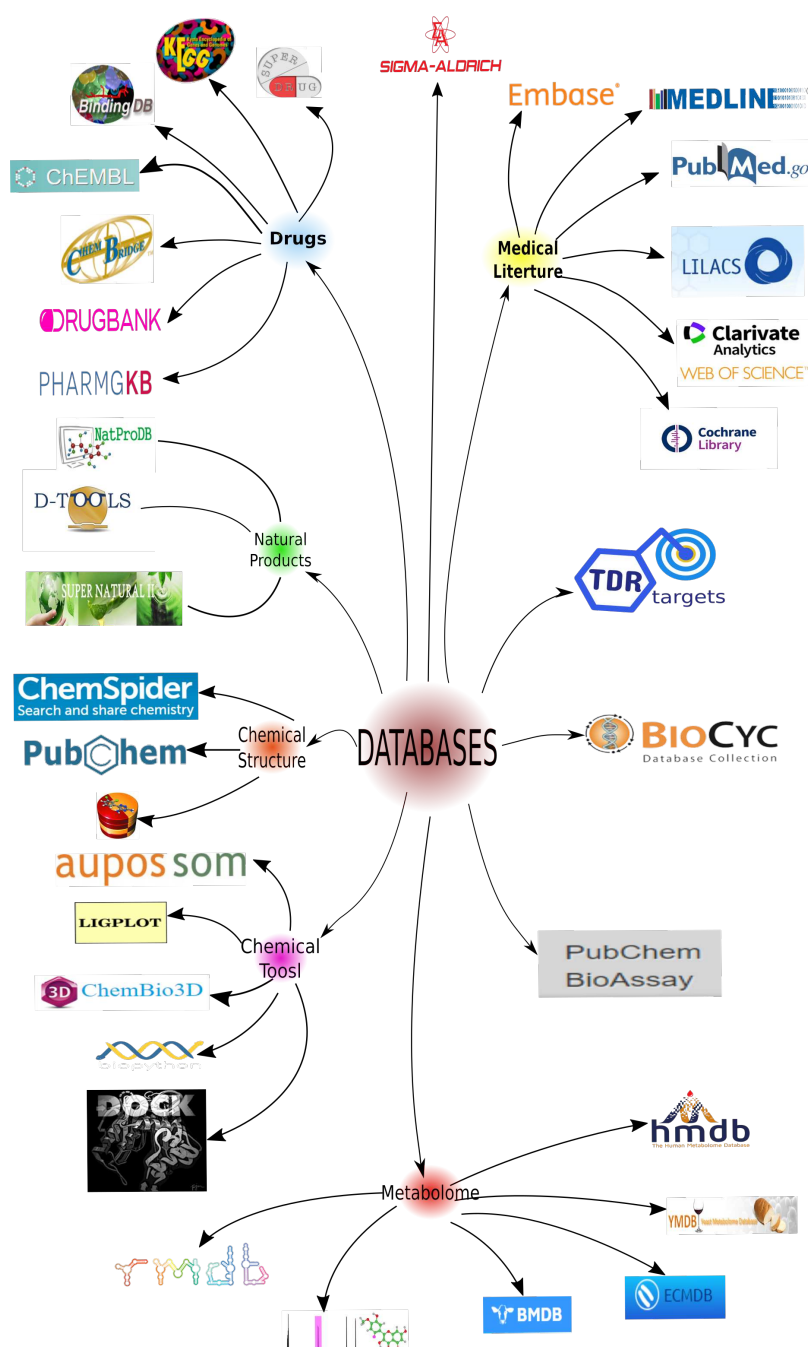


Figure 9. Drug-database review. This figure shows useful information related to general databases and databases related with specific antileishmanial drugs

From Venom to Medicine: Phospholipases A2 (PLA2) as Potential Antiparasitic Agents against Leishmania Parasites

Phospholipase (PL) enzymes constitute a diverse protein group that hydrolyze ester bonds within phospholipid structures. Phospholipids are located in cellular membranes and play important roles in biochemical signaling and lipid metabolism processes. PLs are classified into several families, with PLA, PLB, PLC, and PLD being the most relevant. Specifically, the PLA2 group hydrolyzes the sn-2 position of phospholipids such as phosphatidylcholines and phosphatidylethanolamines, releasing free fatty acids. PLA2 enzymes have been characterized across a wide range of organisms and play conserved roles in lipid digestion and cell signaling cascades through arachidonic acid release. Further

research into the structural biology and biochemical functions of PLA2 enzymes may provide insights into their potential as therapeutic targets or tools. [155,156]

Moreover, the secretory PLA2 (sPLA2) isoforms are the most studied, playing a crucial role in cellular signaling due to their capability to generate free arachidonic acid and important eicosanoid precursors. These eicosanoids regulate inflammatory responses. However, PLs are not only essential for cellular signaling and lipid homeostasis. Some species utilize PLs for alternative functions. For instance, bees and wasps use PLs as venom components to trigger inflammatory and allergic responses in humans. Additionally, certain fungal PLAs facilitate pathogen invasion and damage of host cells during infection. Likewise, in plants, PLA2 contributes to pathogen defense and stress response, enabling plants to adapt and survive adverse conditions. [156–162]

It is important to note that while secondary metabolites like venoms can be harmful, they may also have beneficial functions such as protection against pathogens, symbiotic interactions, or chemical communication. However, venom production is primarily associated with defense and capturing prey. In this context, PLA2 enzymes are key components in snake venoms, aiding in prey digestion and exerting toxic effects. The presence of PLA2 in snake and other animal venoms has sparked extensive research to better understand their mechanisms of action and pharmacological properties for potential drug development. Given their antiparasitic effects against various parasites, PLA2 represents a promising source of antimicrobial peptides (AMPs). For instance, PLA2 enzymes have demonstrated antiparasitic activity against malaria, Chagas disease, and toxoplasmosis parasites. Specifically, they can inhibit parasite growth, disrupt cell membranes, and reduce invasiveness. These examples highlight the diversity of parasites vulnerable to PLA2 and their ability to exert antimicrobial effects through multiple mechanisms. Further research into PLA2 as a source of AMPs could uncover novel therapeutic approaches against parasitic diseases.[157–159,163–165]

Research in this field remains active and focuses on better understanding the mechanisms of action of these enzymes and their potential as therapeutic targets. Advances in this regard could provide new strategies for the treatment of parasitic diseases and contribute to improving global health. The ongoing research and development of therapies based on PLA2 offer hope for effectively combating these parasites and providing more effective and safer therapeutic options in the future.

6. Conclusions

The use of standard drugs in the treatment of leishmaniasis can present certain drawbacks. These drugs may potentially lead to adverse effects, resistance, and toxicity. Furthermore, their administration necessitates regular clinical and paraclinical monitoring, and they can be costly and ineffective in some cases. Consequently, it becomes imperative to conduct studies aimed at identifying the metabolic pathways of the parasite. Such investigations are crucial for pinpointing the vulnerable targets within the parasite, including proteins involved in immune responses and defense mechanisms. By comprehending these specific points, we can develop more efficient treatment strategies that ultimately enhance patient outcomes.

Supplementary Materials: The following supporting information can be downloaded at: http://www.mdpi.com/xx/1/5/https://drive.google.com/file/d/15X1MM5vksSNiQmn-CLO-Txy_SXAcRIAr/view?usp=sharing, Table S1 and Table S2.

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