Chapter

Use of Cell Biology to Identify Cellular Targets in Drug Development Process against *Leishmania* Sp.

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Abstract

Leishmaniasis is one of the most important neglected tropical diseases. The chemotherapy for its treatment uses very toxic compounds with a low efficacy rate. Thus, there is an urgent need to develop new chemotherapeutic agents to help countries control this devasting disease. In drug development, different approaches can be used to identify potential cellular targets that allow us to understand better the cell biology of eukaryotic cells. Several groups are dedicated to studying new molecules, searching for promising candidates against *Leishmania*. Different techniques have been used to characterize the cell biology, biochemistry, and molecular biology alterations induced by the treatments, trying to understand the mechanisms of action. The main goal of this chapter is to describe an overview of the literature exploring the several studies published about the chemotherapy of anti-*Leishmania* concerning the mechanisms of action of different classes of molecules or therapeutic alternatives.

Keywords: chemotherapy, drug development, cell biology, ergosterol, histone deacetylases, organometallic compounds, therapeutic combination, nanotechnology

1. Introduction

Leishmaniasis is a neglected tropical disease that comprises a large and complex group of infections caused by the *Leishmania* genus protozoan parasites. *Leishmania* parasites have intrinsic biological features that make chemotherapy challenging, presenting high adaptability and plasticity. Its life cycle has two different developmental stages: intracellular amastigotes that live in the mammalian host cells and promastigotes that develop in the insect vectors (**Figure 1**) [1].

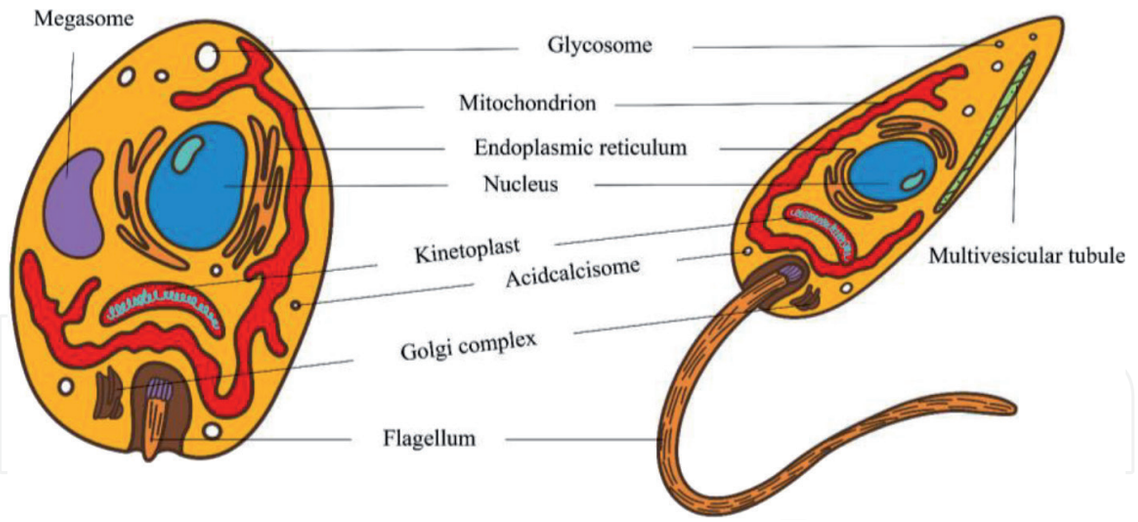

Figure 1.
Illustration of the amastigote (left) and promastigote (right) forms of Leishmania *sp. showing the main organelles and structures.*

The ultrastructure of *Leishmania* parasites (**Figure 2**) presents some conserved features and a classical internal organization of eukaryotic cells, with an individualized nuclear envelope, a single and ramified mitochondrion, endoplasmic reticulum, and Golgi complex, which can lead to difficulties in the development of *Leishmania*-specific drugs with low toxic side effects to mammalian hosts [1]. In addition, however, these protozoans have essential and exclusive organelles and structures such as acidocalcisomes, glycosomes, megasomes, and subpellicular microtubules (**Figure 1**) that can be exploited as drug targets [2].

The *Leishmania* plasma membrane comprises a lipid bilayer associated with proteins and a glycocalyx consisting of a myriad of glycoconjugates. The lipid bilayer has a trilaminar aspect with about 9 nm of thickness. The lipidic composition of the Trypanosomatidae family members is dependent on genus and species. In general, *Leishmania* has 24-methylated sterols, such as episterol, 5-dehydroepisterol, and traces of ergosterol as the major endogenous sterols' constituents, which are absent in mammalian host cells, where cholesterol is the main source of membrane sterols [3]. This divergence in sterol profiles has been exploited to develop drugs that affect the sterols biosynthesis pathway, including azoles,

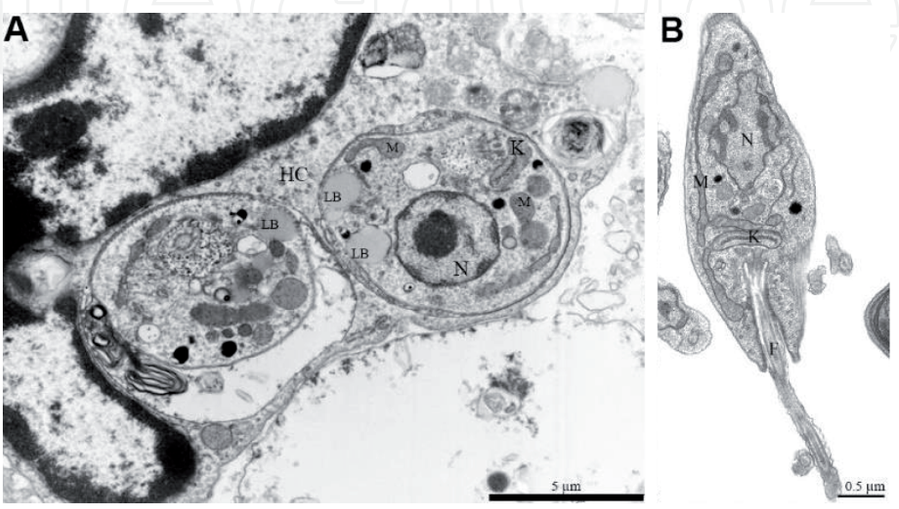

Figure 2.
Ultrathin sections of L. amazonensis *amastigotes (A) and promastigote (B). F, flagellum; HC, host cell; K, kinetoplast; LB, lipid body; M, mitochondrion; N, nucleus.*

azasterols, and others [2]. In addition, many attempts to develop drugs targeting *Leishmania* glycocalyx have also been performed [4].

Leishmania parasites present a single and ramified mitochondrion frequently associated with the plasma membrane, subpellicular microtubules, and endoplasmic reticulum. As well as in other eukaryotes, *Leishmania* mitochondrion operates in energetic metabolism, compartmentalizing the Krebs cycle and performing cellular respiration. The amphotericin B and pentamidine, two of the currently used drugs for the treatment of leishmaniasis, target mitochondria, resulting in a decrease of the mitochondrial membrane potential. Moreover, other drugs targeting mitochondria, such as hydroxynaphthoquinones, have been evaluated against *Leishmania* [5].

To maintain its morphological structure, *Leishmania* has a cytoskeleton composed mainly of subpellicular microtubules, which are filaments finely associated to plasma membrane inner leaflet, regularly spaced, and longitudinally oriented throughout the parasite's cellular body. Despite the phylogenetic conservation of α and β-tubulins, structural divergences in specific tubulin drug binding sites have suggested these proteins as a potential target, as described for podophyllotoxin derivatives and others [6, 7].

In the Medicinal Chemistry field, several approaches have been attempted, trying to find potential cellular targets for developing anti-*Leishmania* drugs. First, nanotechnology-based drug delivery systems have been applied, improving efficacy and enhancing pharmacokinetics properties of currently available drugs [8]. Second, molecular hybridization techniques can combine two drugs or chemical groups with previously known biological activity, producing a single and novel molecule with increased activity [9]. Finally, another alternative is the transposition of a drug already used to treat another disease; it is the case of miltefosine, the last treatment included in the arsenal of chemotherapeutic agents against leishmaniasis. Miltefosine was initially designed as an anticancer medicine, and in 2002 it was registered as first-line treatment, mainly for visceral leishmaniasis (VL), in Asia, Africa, and some regions of Europe.

2. Challenging the target: phospholipid and ergosterol biosynthesis

The first metabolically stable analogs derived from lysophosphatidylcholine were synthesized in the late 1960s. Two decades later, Eibl and Unger synthesized the first alkyl phospholipids (APLs), also called miltefosine, first administrated by an intravenous route to treat systemic tumors [10]. However, the treatment failed, and miltefosine was evaluated against topical cutaneous metastases from breast cancer [11]. In the late 1990s, miltefosine was assessed *in vitro* against the *Leishmania* genus [12, 13] and in murine models infected with *L. donovani* and *L. infantum* [14]. More recently, miltefosine was also evaluated against murine models of cutaneous leishmaniasis, alone or in combination with paromomycin [15–17].

After the evidence of the excellent anti-*Leishmania* activity of miltefosine demonstrated *in vitro* and *in vivo*, clinical tests began to be carried out immediately. Thus, miltefosine was the first oral drug approved for the treatment of visceral leishmaniasis, and for several years it was used as the first choice for the treatment of visceral leishmaniasis (VL), mainly in India [13, 18].

Miltefosine was also evaluated in patients infected with cutaneous leishmaniasis in Colombia, Guatemala, and Brazil [19, 20]. The efficacy in this clinical manifestation was variable, depending on the species. For patients infected with *L. panamensis* in Colombia, the cure rate was 91%, while in Guatemala, the cure rate was 53% for infections with *L. braziliensis* and *L. mexicana* [19]. Furthermore, in Brazil, in patients infected with *L. braziliensis*, the cure rate was 75%, compared to 53% cure achieved with pentavalent antimonials [20].

Several studies have demonstrated that the primary target of miltefosine is the cell membranes, affecting cellular processes such as signal transduction, lipid metabolism, and calcium homeostasis [21]. The selectivity for the plasma membrane is related to its chemical structure formed by a polar choline head bound to a long non-polar hydrocarbon chain, which easily inserts into the lipid bilayer, presenting detergent properties that lead to cell lysis in high concentrations [10]. In *L. donovani* and *T. cruzi*, miltefosine inhibits the phosphatidylcholine biosynthesis pathway (Greenberg pathway), being more selective for the protozoan parasites than mammalian cells, where the main route for phospholipid synthesis is the Kennedy pathway [22, 23]. Miltefosine also interfered with the ergosterol biosynthesis and promoted a disturbance in GPI synthesis [21, 24–26], leading to membrane permeability and fluidity changes. In addition, miltefosine interfered with the host immune response by inducing the production of interferon γ cytokine, leading to a biased immune response towards Th1, which would be a beneficial outcome for immunosuppressed patients [26, 27]. About the ultrastructure of *Leishmania* treated with miltefosine (**Figure 3A**), some studies revealed that it induced several alterations, mainly observed in the mitochondrion, in the plasma membrane, an increase of autophagic structures and phenotypes related to cell cycle arrest and apoptosis-like cell death [26, 28, 29].

With the success of miltefosine, several groups worldwide began to study new chemical routes to synthesize ether phospholipid derivatives in searching for novel molecules more active and selective against *Leishmania* [30]. Our group studied a novel hybrid derivative called alkyl phosphocholine-dinitroaniline, which presented a potent effect against *L. amazonensis* at least around 15-times better than miltefosine [31].

Another essential metabolic route for *Leishmania* and other protozoan parasites is the biosynthesis of ergosterol (or 24-methyl sterols) [32]. *Leishmania* has in these cell membranes three major sterol components that are absent in mammalian cells, 5-dehydroepisterol, episterol, and ergosterol. In mammals, cholesterol is the principal sterol present in cell membranes. Thus, the differences between some steps and enzymes in the biosynthetic route of the protozoan parasites and mammalian host cells have been exploited as targets to develop novel drugs as candidates to chemotherapeutic agents [32].

At least 20 metabolic steps are necessary to synthesize ergosterol, and several enzymes participate in these reaction sequences [32–34]. Furthermore, several works have shown that multiple classes of compounds targeting 24-methyl sterol biosynthesis exhibit suitable anti-trypanosomatid activities *in vitro* (**Figure 3B, C**) and *in vivo* [33–39].

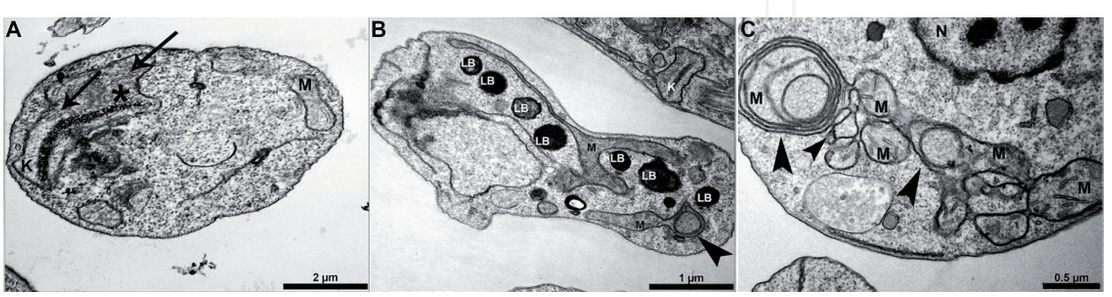

Figure 3.
Ultrathin sections of L. amazonensis *promastigotes treated with miltefosine and two ergosterol biosynthesis inhibitors. (A) 30 µM miltefosine for 72 h; (B) 3 µM ravuconazole for 48 h; (C) 1 µM posaconazole for 48 h. Mitochondrion was the organelle more affected in all treatments, presenting alterations in the cristae and kDNA structure (asterisk, thin arrow, and arrowheads) and mitochondrial swelling and disorganization of its ultrastructure. In panel C, several lipid bodies appeared after treatment. K, kinetoplast; LB, lipid body; M, mitochondrion; N, nucleus.*

More than 30 drugs have been studied in the last 30 years. These drugs are included in large classes of inhibitors, such as 1) Statins, which inhibit the enzyme 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase), also evaluated as cholesterol-lowering drugs; 2) Bisphosphonates that act in the enzyme farnesyl pyrophosphate synthase and inhibit the isoprenoid pathway, mainly used to treat hypercalcemia; 3) Quinuclidines and zaragozic acid, developed to inhibit the squalene synthase, the enzyme that catalyzes the first committed step in the sterol biosynthesis pathway. This class of drugs was developed as an alternative to statin use because they do not inhibit the synthesis of the isoprenoids. 4) Allylamines, which include the known antifungal terbinafine that inhibits the squalene epoxidase; 5) Azoles, which are essential medicines to treat many fungal diseases and inhibit the C14α-demethylase. Several azoles were developed, always trying to find new tolerate and efficacy drugs, also searching to novel molecules to solve the problems with antifungal resistance; finally, 6) Azasterols, which inhibit the enzyme $\Delta^{24,25}$ sterol methyltransferase absent in mammalian cells, one enzyme that catalyzes the methylation of steroid nucleus of sterols, producing 24-methyl sterols, essential for *Leishmania*, *T. cruzi* and fungi (Reviewed in [32]).

In summary, several works have pointed to the importance of looking for the biochemical properties of each enzyme involved in the pathway and its relevance as an essential target for the parasite viability; this feature characterizes the enzyme as a promising target for the development of potential chemotherapeutic candidates for the treatment of leishmaniasis.

3. Challenging the target: histone deacetylases

Histone deacetylase (HDACs) inhibitors are a relatively new class of potential agents in treating neurodegenerative diseases, various types of cancer, and parasitic infections. HDACs have broad importance in the cellular environment. They regulate histone and non-histone proteins affecting the cell cycle, energy metabolism, and inducing cell death. Some HDAC inhibitors were already approved by the FDA (Food and Drug Administration) to treat lymphoma and myeloma, such as vorinostat, romidepsin, belinostat, and panobinostat, in combination with bortezomib and dexamethasone [40]. Given the results obtained *in vitro* and *in vivo* in several disease models, the advancement of clinical trials in tumors, and the transposition of drugs as an old ally in the treatment of leishmaniasis, HDAC inhibitors are a promising approach in the understanding of cell biology of the parasite, especially concerning its chemotherapy.

There are 18 histone deacetylases in humans, which can be grouped according to cell location and the molecule used as a cofactor for its enzymatic action. These HDACs are divided into 1) zinc-dependent HDACs, also called "classical" histone deacetylases; and 2) nicotinamide and adenine dinucleotide [NAD^+]-dependent HDACs. The first one comprises class I (HDACs 1–3, 8), IIa (HDACs 4, 5, 7, and 9), IIb (HDACs 6 and 10), and IV (HDAC 11). While the second one, the NAD^+-HDAC, belongs to class III and is also known as sirtuins (SIRT 1–7). HDACs are still poorly understood and characterized in *Leishmania*. So far, four Zn^{2+}-dependent histones deacetylases and three NAD^+-dependent histones deacetylases were discovered in the parasite [41, 42]. Although four homologs of classical HDACs were identified, none was functionally characterized. Among the information in the literature, a *L. major* HDAC (gene LmjF21.0680) was shown to be expressed during the differentiation of promastigotes to amastigotes, with a possible role in chromatin structure and impacts on gene transcription [43]. Furthermore, Prasanna et al. [44] managed to isolate, express, and purify an *L. donovani* histone deacetylase

(LD_HDAC), with less than 40% identity with class I human HDACs. Information about classical HDACs in the *Leishmania* genus is still very early and deserves further development.

Unlike classical HDACs, there are several studies about NAD^+-dependent HDACs in the *Leishmania* genus. Three sirtuins were already found in *Leishmania*, SIR2RP1, SIR2RP2, and SIR2RP3 [45]. These sirtuins were not found in the nucleus, as described for *Saccharomyces cerevisiae* [45]. SIR2RP1 is expressed from a single copy gene in *L. amazonensis* (LaSIR2RP1), resulting in a monomeric protein with NAD^+-dependent deacetylase action immunodetected in its glycosylated form [46]. LaSIR2RP1 has dispersed localization in the cytosol or cytoplasmic granules [47] and is secreted in lesions derived from intracellular amastigotes [48]. In addition, the *L. donovani* SIR2RP1 was 46% similar to the human SIRT2 [49]. In *L. infantum* and *L. major*, the SIR2RP1 proteins present two functional sites for NAD^+-dependent deacetylation activity and ADP-ribosyl transferase activity [49].

In *L. major* SIR2RP1, removing the acetyl group of lysine 40 from α-tubulin was demonstrated *in vitro* and *in vivo* [50]. However, in these parasites, the ribosylation function of α-tubulin ADP was also shown, resulting in its depolymerization or even inhibiting its assembly [51]. Thus, sirtuins in *Leishmania* have demonstrated an essential role in cytoskeleton dynamics and may have significant implications for remodeling the parasite's morphology and its interaction with the host cell. LmSIR2RP1 also showed a close relationship with the HSP83 protein, an orthologous chaperone of the human HSP90 chaperone, although the intracellular levels of LmSIR2RP1 do not influence the acetylation status of HSP83 [52]. The use of geldanamycin, an HSP90 inhibitor, induced alterations in the cytodifferentiation of promastigotes to intracellular amastigotes [53]; the same was observed for protozoa overexpressing or knockout for the LmSIR2RP1 gene [52]. This study confirms that the SIR2RP1/HSP83 interaction may play an essential role in the differentiation of the parasite.

The work in [54] demonstrated that *L. major* sirtuins may be related to the success of infection through interaction with macrophage surface proteins and, therefore, play a regulatory role in immune responses [55]. This work also showed the capacity of *L. major* sirtuins to trigger the effector response of B cells, promoting a robust humoral response with the secretion of specific antibodies, such as IgG1 and IgG2a [55]. Meanwhile, the overexpression of *L. major* sirtuin, also observed for *L. infantum* sirtuin [48], revealed that it would be involved in the proliferation rate, besides participating in the regulation of death factors, thus preventing death by apoptosis.

The SIR2RP2 present in *L. infantum* mitochondrion is related to the growth rates of promastigote forms with a direct relationship with NAD^+ homeostasis [56]. In 2017, a study revealed that the *L. donovani* SIR2RP2 also has a mitochondrial location [49]. The deletion of this gene led to a reduction in the proliferation rate, further resulting in the interruption of the cell cycle in the G2/M phase. Furthermore, the deletion of LdSIR2RP2 resulted in greater susceptibility of the parasite to commercially available sirtuin inhibitors and a reduction in the mitochondrial membrane potential, resulting in a low concentration of ATP in the mitochondrion. Interestingly, in this SIR2RP2 knockout *Leishmania*, there was an intensification of glycolysis that could be an attempt to compensate for the disbalance of the mitochondrial metabolism. LdSIR2RP2 showed NAD^+-dependent ADP-ribosyl transferase activity with 39% similarity to SIRT4 from humans [49].

The third sirtuin, SIR2RP3, still has few descriptions in the literature. The *L. donovani* SIR2RP3 was 37% similar to the human SIRT5 [49], so that molecular coupling assays, using known inhibitors and SIR2RP3, revealed a strong analogy with SIRT5, which refers to the form of interaction with inhibitors. However, the

interaction of these inhibitors with SIR2RP3 also demonstrated significant molecular differences compared to SIRT5 in humans, which could act as selective targets for the treatment of leishmaniasis [57].

A recent study with a histone deacetylase inhibitor *in L. amazonensis* revealed the sirtuins' potential to develop novel molecules with anti-*Leishmania* activity [58]. Furthermore, this study from our group demonstrated the potent inhibition of parasite proliferation that is probably related to essential functions for HDACs in *Leishmania*, which include the control of the cell cycle and the induction of cell death [58]. Other effects already observed with HDAC inhibitors are different levels of chromatin compaction, increased number of lipid bodies randomly distributed throughout the cytosol, increased production of reactive oxygen species, changes in *Leishmania* morphology, and increased expression of acetylated α-tubulin (**Figure 4**) [58].

However, the parasite's ability to modulate the histone deacetylases of the mammalian host to establish the infection has already been observed. For example, the upregulation of the macrophage HDAC1 was observed during infection with *L. amazonensis*, resulting in deacetylation of the histone tail of the gene's promoter region responsible for producing nitric oxide. This deacetylation prevents the access of transcription factors, which culminates in the repression of nitric oxide production, which allows the establishment of intracellular amastigote forms in the parasitophorous vacuole [59].

Besides, HDAC inhibitors have also been used in combination therapy to treat antimony-resistant *L. donovani* infection, where the upregulation of the multiresistant protein depends on IL-10 production. Thus, the imipramine antidepressant positively regulates HDAC11, inhibiting the acetylation of the IL-10 promoter, which leads to a decrease in its production [60]. This imipramine-mediated lowering of the IL-10 level reduces MDR-1 expression and aids in the elimination of the parasite.

Thus, histone deacetylase inhibitors belong to a class of compounds with potential application to develop novel molecules with anti-*Leishmania* activity and for the treatment of leishmaniasis, alone or in combination with other medications already established. Furthermore, the World Health Organization (WHO) has already recommended the therapeutic combination to reduce the doses and toxicity and develop a more effective and safe treatment. Finally, HDAC inhibitors seem to be an exciting tool for a better understanding of the Cell Biology of *Leishmania* and enabling the knowledge of new routes for the development of novel drug candidates.

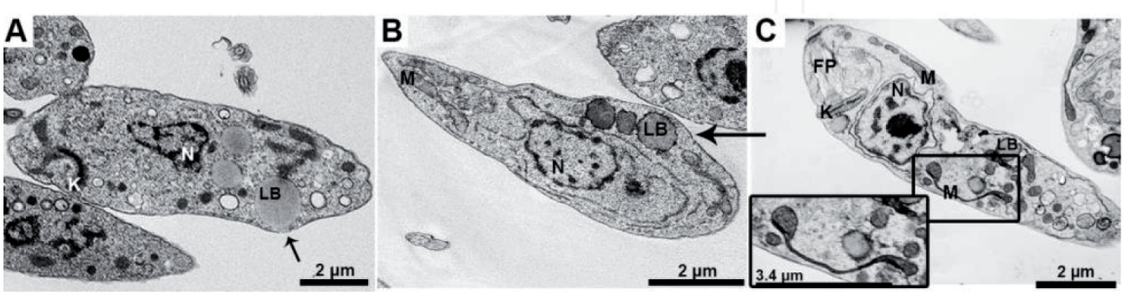

Figure 4.
Ultrathin sections of L. amazonensis *promastigotes treated with HDAC inhibitors for 48 h. (A) 1.5 µM NIH119; (B) 1 µM NIH119; (C) 15 µM tubastina A. NIH119 and tubastatin a induced different ultrastructural alterations such as 1) increase in the number of lipid bodies, some of them presenting different morphologies (A, B); 2) presence of membrane protrusions (A, B – Arrow); 3) alterations in the kinetoplast (A); 4) decondensation of chromatin (B, C); and, 5) changes in mitochondrial ultrastructure. FP, flagellar pocket; K, kinetoplast; LB, lipid body; M, mitochondrion; N, nucleus.*

4. Challenging the target: organometallic compounds

Although metals have been used in Medicine for centuries, most compounds produced by the pharmaceutical industry are still based on organic molecules. Nevertheless, new perspectives about metal-based drugs and their therapeutic potential against cancer, bacteria, virus, and even trypanosomatid infections have emerged in the last few years. In this context, *Leishmania* infections have been treated with pentavalent antimonials since the 1940s [1], which indicates that new compounds containing other kinds of metals may present anti-*Leishmania* activity, thus opening the possibility to reduce the toxicity based on the metal-drug synergism.

Platinum-derived metals are well known to have antitumor effects due to their ability to bind to DNA molecules. So, since tumor cells and kinetoplastid parasites present similar metabolic pathways [61], the coordination of these metals to organic compounds might be efficient in treating *Leishmania* infections. For example, the (2,2′:6′2″-terpyridine)platinum(II) complexes can inhibit 100% of the growth of *L. donovani* amastigotes at a concentration of 1 μM through the intercalation of terpyridine and platinum(II) into the DNA, probably binding to guanine bases or some enzyme active sites [62]. More recently, cisplatin-derived complexes (*cis*-diamminedichloroplatinum(II)), an anticancer drug, were tested on *L. infantum* promastigotes and amastigotes, revealing a remarkable anti-*Leishmania* activity with IC_{50} values of 1.03 μM and 0.10 μM, respectively. Furthermore, the treatment with *cis*-DDP induced loss of mitochondrial transmembrane potential and DNA fragmentation, thus leading to apoptosis-like death [63].

In addition to platinum and its derivatives, other transition metals have been widely studied in terms of antiprotozoal activity. For example, organometallic complexes containing ruthenium(II) and anti-inflammatories were evaluated active against *L. amazonensis* and *L. infantum* promastigotes, presenting IC_{50} values comparable to the meglumine antimoniate, one of the first-line drugs for *Leishmania* infections [64]. Moreover, the coordination of gold compounds with organic ligands was efficient against *L. amazonensis* and *L. braziliensis* promastigotes while presenting low toxicity to host cells [65]. Thus, the therapeutic mechanism of these organometallic molecules may be related to induction of oxidative damage and alterations in the membrane permeabilization by the inhibition of specific membrane protein channels and zinc-binding proteins. These alterations can lead to parasite cell death by apoptosis-like and necrosis.

Among transition metals, the essential ones, such as zinc and copper, are present in cell structures and involved in many cellular processes, becoming indispensable for host–parasite interactions. Zinc regulates gene transcription processes and cell signaling, while copper is also a critical enzymatic cofactor for organ functioning and multiple metabolic processes [66]. Therefore, the coordination of organic molecules to essential metals regarding new antiprotozoal treatments might increase the drug uptake and contribute to the parasite's elimination. The zinc(II)-dipicolylamine (ZnDPA) coordination complexes were active against *L. major* promastigotes *in vitro* with IC_{50} values between 12.7 μM and 0.3 μM and minimal mammalian cell toxicity. The compounds also showed *in vivo* activity, with a high affinity for intracellular amastigotes and low toxicity to mice [67]. The effects of a copper dimethoxy bipyridine (CuDMOBP) complex were also investigated against *L. major*; the complex presented significant *in vitro* activity with high selectivity index [68]. Results from quantitative real-time PCR also indicate a significant reduction in cellular expression of IL-10 and TNF-α in macrophages treated with CuDMOBP, probably due to a reduction of the parasite population.

Metals have also been combined with ergosterol biosynthesis inhibitors, including the azoles family of drugs, which are used to treat fungal infections and present activity against protozoan parasites. For example, a recent study from our group revealed the potent effect of the combination of itraconazole (ITZ) with zinc (Zn) against *L. amazonensis* promastigotes (**Figure 5B**) and intracellular amastigotes [69]. The biological effects were significantly increased when the itraconazole was coupled with zinc, resulting in IC_{50} values in nanomolar ranges and cell death of parasites in low concentrations [69]. Likewise, the coordination of clotrimazole, ketoconazole (**Figure 5A**), and miconazole to manganese (Mn) provide novel molecules with better activity against *L. major* when compared to the original organic antifungals [70].

Despite Medicine's advances in the past decades, information about organometallic drugs is still lacking. More profound studies must be done to understand the role of metals in host–parasite interactions, thereby better comprising the mechanisms of organometallics drugs against the parasites and mammalian host cells. Nevertheless, the literature available indicates that organometallics are a promising class of drugs for treating leishmaniasis.

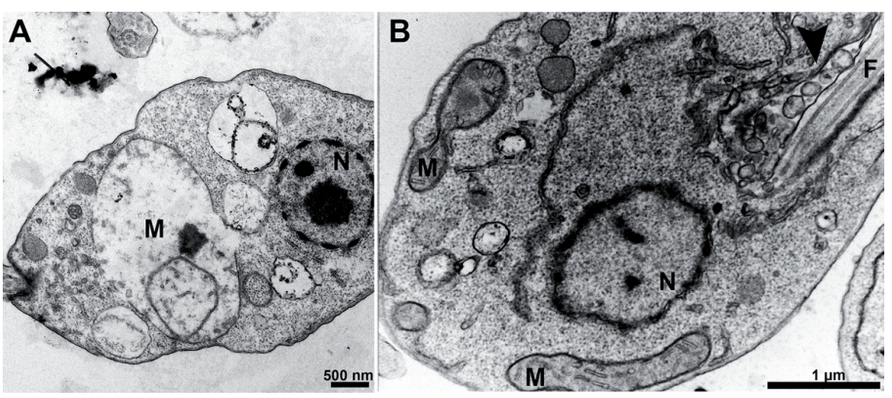

Figure 5.
Ultrathin sections of L. amazonensis *promastigotes treated with organometallic compounds. (A) Treatment with 300 nM ketoconazole-ruthenium induced alterations in the mitochondria, such as swelling and loss of the mitochondrial matrix and the formation of large autophagic vacuoles. (B) Treatment with 0.5 μM itraconazole-zinc resulted in mitochondrial alterations and an increase in the secretion of vesicles (arrowhead) at the flagellar pocket. In both panels, the nuclear chromatin appeared altered. F, flagellum; M, mitochondrion; N, nucleus.*

5. Therapeutic combination: what do we know?

There are many strategies to treat leishmaniasis; however, several studies have shown the numerous advantages of therapeutic combination, like observing for other diseases. For example, combining drugs from different chemical classes could reduce the total drug doses or treatment duration. These aspects are important to minimize toxic side effects, submission at treatment, less load on the public health system and reduce cases of drug resistance. Also, the therapeutic combination could improve treatment efficacy for refractory or complicated cases, such as in patients coinfected with HIV. A successful study conducted by the Drugs for Neglected Diseases initiative (DNDi), in partnership with Médicins Sans Frontières (MSF) and other institutions, pointed to evidence of the high efficacy of the combination therapy to treat patients with visceral leishmaniasis (VL) in coinfection with HIV [71]. Although the current WHO guidelines recommend the treatment of HIV/VL coinfection with liposomal amphotericin B (AmBisome®), this work strongly supports a change in the treatment recommendations, from AmBisome monotherapy to

combination therapy as the first-line treatment. Moreover, they suggest the combination with miltefosine once this combination therapy has a good safety profile and is highly efficacious [71].

Nowadays, combination therapy is an efficient tool to treat many microbial infections such as AIDS, tuberculosis, malaria, and several other diseases. Recent works have shown that combination therapy for leishmaniasis has progressively been recommended to increase treatment tolerance and efficacy, reduce cost and treatment duration, and limit the growth of drug resistance [72–75]. For the treatment of leishmaniasis, WHO has recommended combination therapy based on many studies showing the efficacy of this therapeutic tool; the combinations include novel synthesized drugs, nanoparticles developed for drug delivery, repositioned drugs, old medicines, and immunomodulatory agents [76–79]. Indeed, several studies have reported the superior efficacies of combination therapies against leishmaniasis. Some of them demonstrated the synergic effects of combinations between amphotericin B with other available medicines, such as meglumine antimoniate, miltefosine, paromomycin, or azithromycin [80–82].

Analysis of drug interactions aimed to show if the interaction between them is classified as synergistic, antagonistic, or indifferent. *In vitro* data are based on an extended ratio and concentration range. However, *in vivo* combinations are more complex and less defined, with the number of doses limited. The synergy between two (or more drugs) occurs when their combined activities are improved over the sum of their separate individual effects. Synergistic drug combinations provide lower concentrations of both compounds, enhancing therapy outcomes by increasing efficacy and reducing side effects. Moreover, synergistic combinations could reintroduce those that have lost activity against drug-resistant strains. The FICI (fractional inhibitory concentration index) value is considered the standard reference parameter to quantify interactions between pairs of drugs. Odds in 2003 [83] established more restrictive criteria to analyze experiments and defined "synergy" as a $\geq$ 4-fold reduction in the MICs of both compounds in combination when compared to their MICs alone, where the FICI value must be $\leq$0.5. MIC means the minimum inhibitory concentration, i.e., the lowest drug concentration with no visible cell growth.

In vitro studies against *L. amazonensis* suggest that combinations between compounds that act in different biosynthetic pathways of the parasite, such as sterol biosynthesis, are promising [32]. Interestingly, a recent study showed that sterol biosynthesis inhibitors and alkylphosphocholine analogs, combined with medicines available to treat other diseases, are efficient against trypanosomatids [32, 36, 78, 84]. Besides, another study showed the *in vivo* efficacy of the combination therapy between miltefosine, an alkylphosphocholine, and amphotericin B or paromomycin [85], a therapeutic alternative to treat antimony-resistant VL cases in India. Furthermore, topical treatment against cutaneous leishmaniasis might be effective when amphotericin B and miltefosine are co-loaded at second-generation ultra-deformable liposomes since *in vivo* studies displayed a significant reduction in the parasitic burden [86]. However, a Brazilian survey against *L. infantum* revealed a decrease of the miltefosine concentration when combined with lopinavir (anti-HIV drug); yet, the synergistic effect was not evidenced [87]. Unexpectedly, the combination of nelfinavir with miltefosine presented better results, with FICI $\leq$0.5. Thus, this study also concluded that the combination might be helpful to treat patients with visceral leishmaniasis who also are infected with HIV [88].

Combination therapy is a promising strategy to treat several diseases. Therefore, it is urgent to investigate synergistic and other drug combinations to increase novel probabilities of therapeutic protocols to treat leishmaniasis. The discovery and the analysis of drug combinations can be facilitated by the collective use of different

approaches and methods. Drug combinations have proved to be a successful strategy to shorten the course of therapy and reduce toxicity through lower dosage administration; these strategies should reduce the appearance of new resistant parasites. Thus, recent proposals of combinations have been suggested as state-of-the-art for the treatment of leishmaniasis. In the short run, combination therapy is an interesting way to improve the treatment for leishmaniasis.

6. Where are we going? Nanotechnology

Recent advances in Nanotechnology have had a profound impact on health sciences, especially Medicine, because of the development of different nanomaterials designed as intracellular carriers to deliver drugs and genes. The development and use of nanocarriers have also been established in the field of Pharmaceutical Sciences by enabling the encapsulation of drugs creating stable and controlled environments, and improving the biocompatibility of these drugs in various biological systems. These nanocarriers were developed to improve the solubility of poorly soluble drugs, control or maintain their release, and protect them from degradation. These characteristics increase drug bioavailability, reduce systemic side effects, and increase drug specificity for biological targets. For these reasons, Nanotechnology is a new field that allows the construction of versatile diagnostic and therapeutic platforms using nanocarriers as molecular machinery for different clinical applications.

The development of nanocarriers began in the 1960s to always seek to improve biocompatibility and reduce the toxicity of nanomaterials. The second generation of nanocarriers was developed around the 1980s and sought to improve the surface of these materials by increasing their stability, stealth, and targeting ability. The third generation of nanocarrier introduced the idea of intelligent nanomedication to enhance the targeting mechanisms and theranostic capabilities of these nanomaterials [89]. The word *nanoparticle* has been widely applied to describe numerous pharmaceutical carriers or imaging systems based on nanoscale materials. Nanoparticles are particulate materials in their solid or dispersed state present in a size scale between 10 nm and 100 nm (ISO/TS 80.004–1:2015). Due to the great diversity of these nanomaterials, the scientific community elaborates a classification based on their characteristics and properties.

Initially, the nanomaterials are divided into two classes: inorganic and organic. However, they are divided into three subgroups, according to some of their characteristics. The first would concentrate single-chain polymer-drug conjugates, polymeric colloids prepared by techniques such as emulsion polymerization, cross-linked nanogel matrices, dendrimers, and carbon nanotubes, where the nanocarrier is a single synthetic molecule with covalent bonds and a relatively large molar mass. The second subgroup of nanocarriers would comprise self-assembly of smaller molecules such as 1) liposomes and polyplexes, the most studied members of this group of nanoparticles; 2) polymersomes and other sets of block copolymers; 3) colloidosomal aggregates of latex particles and sets of proteins or peptides. In this case, the dynamic nature of these types of systems depends on intermolecular forces and biological conditions. Finally, the last subgroup of nanocarriers would include complexes based on fullerenes, silica, colloidal gold, gold nanoshells, quantum dots, and superparamagnetic particles.

Another critical point in developing nanocarriers is the synthesis, which can be rationally divided into two fundamental stages: nucleation and growth. Understanding and manipulating these two steps have created new possibilities allowing researchers to easily control the synthesis of nanoparticles in terms of

size, morphology, and monodispersity. The choice of the synthesis route provides a characteristic set of advantages and disadvantages in nanomaterial production.

In *Leishmania* sp., the use of nanocarriers has been studied since the late 1970s with the development of liposomal amphotericin B [90, 91]. Since its development, liposomal amphotericin B has become more efficient and bioavailable, less toxic, and better tolerated by patients [92, 93]. This formulation also has a broad and rapid biodistribution reaching steady-state plasma concentrations faster with higher total plasma concentration when compared to its deoxycholate form. Furthermore, liposomal amphotericin B is probably inactive because it is fixed to the liposome; thus, the biologically active drug is released only after direct contact with protozoa or fungal cell walls [94]. In 2010, the WHO proposed the administration of liposomal amphotericin B.

The success achieved by the liposomal formulation of amphotericin B is related to its properties as a nanocarrier system, which has numerous advantages. However, despite these advantages, this system has some disadvantages, including its high cost, limiting its use [94]. Thus, the development of new, cheaper, and more efficient nanoformulations is necessary. Furthermore, different nanocarriers have been developed in recent decades, searching for new therapeutic alternatives to treat leishmaniasis, including nanoparticles based on liposomal, lipid, polymeric, and metallic nanomaterials [8, 95–98]. Therefore, choosing the correct nanocarriers is crucial to define properties and characteristics for this proposed new therapeutic approach. Thereby, this enormous diversity of available nanoparticles makes the development of nanocarriers for the treatment of leishmaniasis very promising since each of these carrier systems has advantages and limitations over each other [8].

Some nanoparticles have been generating significant repercussions for presenting theranostic properties, thus allowing them to be used simultaneously for diagnosis and therapy. Superparamagnetic iron oxide nanoparticles (SPIONs) are one example of this type of nanomaterials. SPIONs have excellent biocompatibility, degradability in moderate acid conditions, magnetic properties, and the ability to generate heat when subjected to an alternating current magnetic field [99, 100]. In addition, this type of nanomaterial still has a large surface area presenting great chemical diversity, which can increase the efficacy of the treatment. Finally, these nanomaterials can also be conjugated to specific molecules to facilitate selective and efficient drug delivery to a diseased tissue or organ [101]. The application of this type of nanomaterial for the treatment of leishmaniasis has been studied by different groups and has shown promising results over the past few years (**Figure 6**) [102–105].

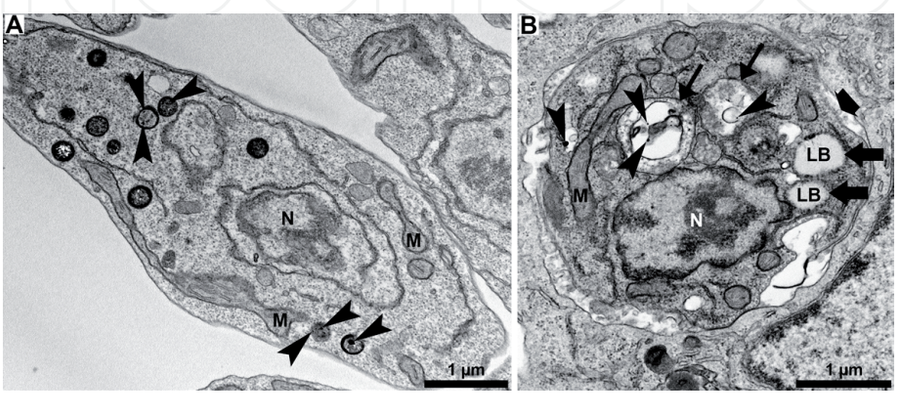

Figure 6.
Ultrathin sections of L. amazonensis *promastigotes (A) and amastigotes (B) after treatment with 100 µg/mL SPIONs for 24 h. In both developmental stages, SPIONs were found inside membrane-bound structures (arrowheads). Some alterations were also observed in the treated parasites, such as increased number of lipid bodies (arrows), presence of autophagosome (thin arrows), and secretion of extracellular vesicles (large arrow).*

In summary, Nanotechnology and the use of nanoparticles inaugurated a new field in health science called Nanomedicine, one of the most promising branches of contemporary Medicine. Thus, the development of new nanomaterials to treat leishmaniasis significantly increases the possibility of finding novel therapeutic alternatives, mainly considering the great diversity of clinical manifestations. An excellent example of this is the use of nanoparticles to develop a topical treatment that can revolutionize the treatment of cutaneous leishmaniasis.

7. Conclusions and perspectives

The treatment of infectious diseases depends on a better understanding of Cell Biology, mainly for parasites that are intracellular obligate eukaryotes, such as *Leishmania*. The knowledge about these parasites allows the identification of essential metabolic pathways and the mechanisms of action involved in drug inhibition, enabling the application of new treatments and conventional treatments combined with new therapies, also using Nanotechnology. Thus, the improvement of tested molecules associated with drug delivery techniques is the path to success for novel leishmaniasis treatments.

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