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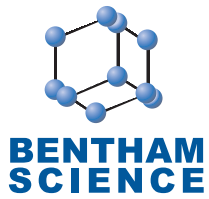

Neglected Tropical Protozoan Diseases: Drug Repositioning as a Rational Option

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Abstract: Neglected tropical diseases represent a major sanitary problem and a huge economic burden to endemic countries, and are currently expanding to non-endemic countries owing to migration currents. Though long abandoned in the past, recent research on novel therapeutics has already started to show results. Drug repositioning is one of the prominent, more successful strategies to approach the development of new treatments for these diseases. Here we present an overview on the limitations of the current available medications to treat African trypanosomiasis, Chagas disease and Leishmaniasis, along with a review on drug candidates presently undergoing clinical trials and drug candidates identified through drug repositioning initiatives.

Keywords: African trypanosomiasis, Chagas disease, Clinical trials, Drug discovery, Drug repositioning, Drug repurposing, Leishmaniasis, Neglected tropical diseases.

1. INTRODUCTION

According to the World Health Organization (WHO), *"Neglected tropical diseases (NTDs) are a diverse group of diseases with distinct characteristics that thrive mainly among the poorest populations"*. They represent a significant threat to health and are responsible for more than a million deaths annually. Although NTDs generate important economic impacts, costing billions of dollars every year, they were largely abandoned in terms of funding, research and policy.

Sleeping sickness (Human African trypanosomiasis), Chagas disease (American trypanosomiasis) and Leishmaniasis, which belong to the NTDs caused by protozoan parasites, are included among the 13 most-widespread diseases in the world [1]. With an initial focus in Africa (Sleeping sickness and Leishmaniasis) and Latin American countries (Chagas disease), they have expanded due to the globalization process that propels migration of people from endemic areas to non-endemic countries, a phenomenon inverse to the one that occurred when Old World diseases were introduced in the Americas during the colonization period. The emergence of new vectors, reservoirs and transmission forms – as blood transfusion and organ transplant - of the parasites causing these infections is another important key in the disease prevalence and spread [2-4].

In the nineties different initiatives in Latin American countries started undertaking surveillance and control of population and vector eradication with successful results [5]. Since the 66th World Health Assembly, in May 2013, the stakeholder countries have signed an agreement *"to strengthen efforts for intensified, integrated measures and planned investments to improve the health and social well-being of affected populations"*. In this frame, different innovative programs aimed at finding novel drugs for tropical diseases are underway [6]. These strategies involve collaborative projects between scientists in academic institutions and/or pharmaceutical laboratories from endemic and non-endemic countries [7].

Known treatments have historically been poorly effective or have become less effective by mechanisms of drug resistance; thus, there is an urgent necessity for therapeutic alternatives. The repurposing of drugs originally licensed for other indications and the rescue of abandoned drugs emerge then as attractive approaches for the development of novel therapeutics for neglected diseases. Drug repurposing

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is a strategy that implies expanding the utility of existing drugs, sometimes generating new formulations (generally with different strengths/combinations/dosing regimens) against a new disease [8].

Drug repurposing and drug rescue strategies, have the potential advantage of facilitating rapid and cost effective drug development because preclinical and clinical development can be built already available data (safety and other pharmaceutically relevant data). Development of new formulations and combinations of existing drugs is also a clever way of extending uses of diverse therapeutics. The reformulation of drugs can improve compliance, pharmacodynamics and pharmacokinetics making them more effective [8-9]. Combination treatments are also believed to prevent the development of drug resistance and for this reason are now recommended standard practice for malaria chemotherapy and other diseases where multiple drugs are available [8, 10-11].

This manuscript reviews the recent advances in the field of drug repurposing for protozoan parasitic diseases, describing the effects of drugs that act against parasites or regulate some specific stages in the host cells that are vital for parasitic survival and / or infection progress.

2. HUMAN AFRICAN TRYPANOSOMIASIS

Human African trypanosomiasis (HAT) or sleeping sickness is one of the most neglected tropical diseases. HAT affects mainly poor people living in 250 rural foci scattered over 36 sub-Saharan African countries where it is a major cause for morbidity and mortality [12]. The disease is caused by the flagellated protozoa *Trypanosoma brucei gambiense* and *T. b. rhodesiense* and is mainly transmitted through the bite of infected tsetse flies. Other less frequent ways of infection include congenital transmission, blood transfusions, laboratory accidents and, probably, sexual transmission [13].

The lifecycle of *T. brucei* has four main developmental stages that occur in the tsetse fly and in the mammalian host, i.e. epimastigotes, procyclic forms, slender metacyclic trypomastigotes, and stumpy metacyclic trypomastigotes. An infected tsetse fly injects stumpy metacyclic trypomastigotes in a mammalian host during the blood meal. These parasites enter the lymphatic system and pass to the bloodstream, they transform into bloodstream trypomastigotes that go to other parts of the body [14]. Later, they enter the Central Nervous System (CNS) and they cause the typical symptoms of HAT: disturbed sleep pattern, confusion, sensory disturbances, extreme lethargy, poor condition and coma [12]. The entire life cycle of *T. brucei* in the mammalian hosts occurs in the extracellular space, completing the parasite cycle when tsetse fly ingest bloodstream trypomastigotes from the infected mammal. In the fly's midgut, they transform into proliferative trypomastigotes that multiply, and after leaving the midgut, they transform into epimastigotes. In the salivary gland, epimastigotes also continue the division and eventually transform into non-proliferative, stumpy, metacyclic trypomastigotes, which are able to infect a new human host and thus continue the cycle [14].

There are two forms of HAT. The prevalent form in Central and West Africa is caused by the sub-species *T. b. gambiense*. In general terms, the parasitemia associated with *T. b. gambiense* HAT remains low, and a chronic infection is established. It takes on average two years before the parasites establish within the CNS and two additional years of progressive deterioration (during stage 2, see the next paragraph) prior to death. In contrast, *T. b. rhodesiense* is found in Eastern and Southern Africa and usually causes a more acute disease, lasting just several weeks to months before death [8].

The HAT clinical features present two stages: in the early stage or stage 1, parasites proliferate in the blood and the lymphatic system and is characterized by indeterminate symptoms such as headaches; in the late stage or stage 2, neurologic parasite invasion of the CNS results in progressive neurological breakdown [15]. Remarkably, the ability of the parasite to invade the CNS makes CNS bioavailability (i.e. the ability of a drug treatment to cross the blood-brain barrier and built up effective drug levels in the brain) a critical aspect to be considered when searching new therapeutic agents.

Disease diagnosis, treatment and management are notoriously difficult. The reasons are numerous and include the fact that HAT diagnostic tools are outdated and often difficult to use in resource-limited settings. For instance, a lumbar puncture is required to establish if a person is in the late neurologic stage of the disease. Similar issues can be evoked in relation to available drug treatments. While, owing to the unfavorable socio-economical background (scarce accessibility to medical care), availability of orally safe and effective medications would be ideal, many of the known treatments are either parenteral drugs or require medical supervision. Other factors include difficulties in accessing known or suspected remote and/or afflicted by violent conflict endemic areas, lack of robust surveillance of old foci, and complex labour- and resource-intensive treatments [16].

HAT is endemic in 36 African countries, occurring in highly specific foci in rural areas. Between 2000 and 2010, 94 HAT cases were reported in non-endemic countries. 72% of which were due to the East-African form (Rhodesiense HAT), and 28% due to the West-African form (Gambiense HAT). Rhodesiense HAT classically occurs in tourists visiting the game parks of East and South Africa. In Gambiense HAT the main risk groups are migrants or long-term travellers [17].

2.1. Drugs and Treatment

There is an urgent need for new drugs for neglected tropical protozoan diseases such as African trypanosomiasis, Chagas disease and Leishmaniasis, which cause more than 120,000 fatalities annually. Some of the poorest areas of the world are afflicted by these vector-borne parasites. Moreover, as introduced above, current treatments for these diseases are not ideal, with issues such as unacceptable toxicity, acquired drug resistance, prolonged hospitalization and high costs [15]. Below these lines we present a summary of available drug treatments for HAT along with their limitations and an overview of drug candidates currently at clinical trials. Available and experimental drugs originating from drug repositioning initiatives are discussed separately in the next sub-section. Table **1** summarizes the approved and experimental drugs (at clinical stage) for the treatment of HAT.

The same structure is conserved for the following sections of the article, dedicated to available and experimental drugs for Chagas disease and Leishmaniasis.

Pentamidine, (4,4-[pentane-1,5-diylbis(oxy)]dibenzenecarbo-ximidamide), is an aromatic diamidine classified as a broad-spectrum antiparasitic drug (Fig. **1**). It has been used for decades against several trypanosomatids, such as *Leishmania major* and *Trypanosoma brucei,* and some fungi such as *Pneumocystis jirovecii*. It has been used to treat first-stage HAT since the 1940s, typically via intramuscular injection. However, it is ineffective against second-stage HAT due to low blood-brain barrier (BBB) permeability [18].

Suramin (Fig. **1**) is perhaps the oldest antimicrobial drug in continuous use since its introduction in 1922. It is the first line treatment for Rhodesiense HAT; drug regimen typically involves intravenous infusion every 3−7 days for a 4-week period. It is highly protein bound and has a very long terminal half-life of 41−78 days; it does not cross the blood-brain barrier. This drug is an effective therapy whose side effects are either mild or reversible, including urticarial rash in about 90% of patients, pyrexia, nausea, and reversible nephrotoxicity. The mechanism of action for Suramin on trypanosomes is unknown. Resistance in the field has been rarely reported [19]. Suramin is also effective against *T. b. gambiense* infections. Because of the risk of sudden shock in case

Table 1. Approved and experimental drugs at clinical stage for the treatment of HAT.

Drugs (way of administration)	Stage disease	*In vitro* trypanocidal activities (IC_{50})	Adverse Effect	References
Pentamidine (IM)	First stage	*T. b. rhodesiense* STIB900 (wild type) 0.002 ± 0.0003μM *T. b. gambiense* STIB930 0.016 ± 0.001 μM	Hyperglycaemia or hypoglycaemia, prolongation of the QT interval on electrocardiogram, hypotension, and gastrointestinal features.	[21, 32]
Suramin (IV)	First stage	*T. b. rhodesiense* STIB900 (wild type) 0.062±0.046 μM	Renal failure, skin lesions, anaphylactic shock, bone marrow toxicity, and neurological complications such as peripheral neuropathy	[21, 31]
Melarsoprol (IV)	Second stage (active against *T. b. gambiense* and *T. b. rhodesiense*)	*T. b. rhodesiense* STIB900 (wild type) 0.011 ± 0.003 μM *T. b. gambiense* STIB930 0.012 ± 0.005 μM	Reactive arsenical encephalopathy (RAE) has been attributed to the toxic effect of Melarsoprol, peripheral neuropathy, cutaneous reactions, renal or hepatic dysfunction, allergic or hypersensitivity reactions	[21, 32]
SCYX-7158 (PO)	First Stage Second Stage	*T. b. rhodesiense* STIB 900 0.294 μg/ml T. b. gambiense 108R 0.165 μg/ml	Not informed	[25]
Eflornithine (IV)	Second stage (only useful against *T. b. gambiense*)	*T. b. rhodesiense* STIB900 (wild type) 8.58 ± 2.7 μM *T. b. gambiense* STIB930 2.85 ± 0.98 μM	Generally, adverse reactions to Eflornithine are reversible after the end of treatment. They include convulsions, gastrointestinal symptoms like nausea, vomiting and diarrhea, bone marrow toxicity leading to anemia, leucopenia and thrombocytopenia.	[32, 35-36]
Nifurtimox + Eflornithine	Second stage considered as an option for Melarsoprol-refractory late-stage disease	Data not available	Convulsions, gastrointestinal symptoms like nausea, vomiting and diarrhea. Genotoxicity, neurotoxicity.	[37-40]
Fexinidazole (PO)	First Stage Second Stage	*T. b. rhodesiense* STIB900 (wild type) 2.17 ± 0.29 μM *T. b. gambiense* STIB930 1.84 ± 1.13 μM	Headache and vomiting of acceptable intensity	[31-32, 41]

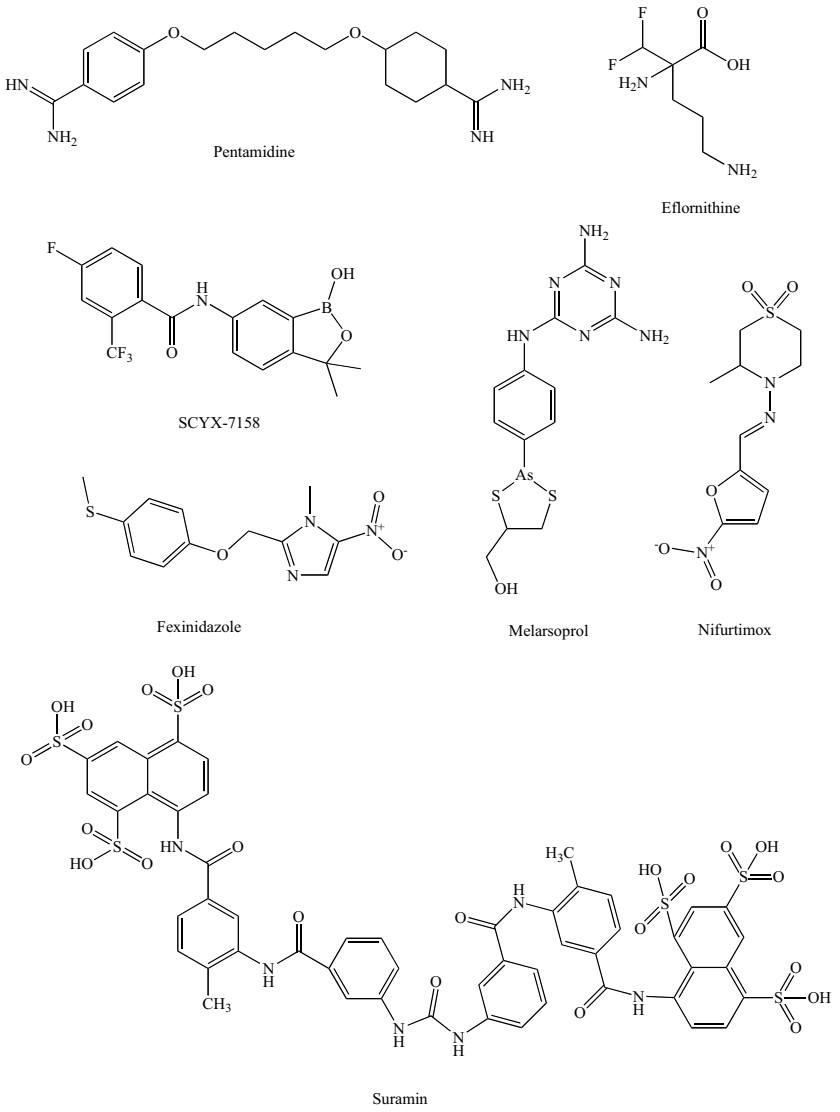


Fig. (1). Molecular structures of approved as well as experimental drugs against HAT at clinical stage of development.

of infection by *Onchocerca volvulus*, treatment with Pentamidine is preferred [20].

Melarsoprol is an arsenical drug used for late-stage HAT (Fig. **1**). Arsenicals were the first drugs introduced for treating sleeping sickness starting with a drug called atoxyl, an ironic name since clinical studies showed it caused blindness due to optic nerve atrophy [21]. Melarsoprol was the first and still the only effective drug for late-stage HAT due to *T. brucei rhodesiense*. It is perhaps one of the most dangerous drugs used for treating an infectious disease due to the presence of the arsenic atom. However, since late-stage HAT is uniformly fatal, medical providers have been forced for decades to accept it as the only therapeutic option. On top of this, this drug is associated with agranulocytosis, peripheral neuropathy, cutaneous reactions, renal or hepatic dysfunction, allergic or hypersensitivity reactions [21-22]. However, Kuepfer *et al.* found that short-term treatments (10 days) are effective with reduced toxicity [23]. Moreover, a combination of Melarsoprol with Cyclodextrin has been proposed as an orally administered, safer alternative [24].

Benzoxaboroles, a novel class of small-molecule boron-containing compounds, which were discovered in 2011 by Jacobs *et al.* [25]. Optimization of a series of such compounds that displayed parasitical activity against *T. brucei* led to the identification of **SCYX-7158** (Fig. **1**), a candidate with potent trypanocidal and attractive physicochemical and ADME -absorption, distribution, metabolism and excretion-properties. In animal models of HAT, SCYX-7158 exhibits significant activity following oral administration, including cure of CNS *T. brucei* infection. The *in vivo* pharmacokinetic characterization of SCYX-7158 reveals that this compound is highly bioavailable across species, and can cross the blood-brain barrier to achieve therapeutically-relevant concentrations in the brain and cerebrospinal fluid of rodents [25]. Based on these properties, SCYX-7158 advanced to preclinical stage, with successful outcomes [26].

SCYX-7158 entered First-in-Human studies in March 2012 and became the DNDi's (Drug for Neglected Diseases initiative) first entity resulting from lead optimization efforts to enter early clinical development. The Phase I study in healthy volunteers of sub-Saharan origin was temporarily

halted in 2013 after the first dose of SCYX-7158 showed a longer than expected half-life in human plasma, triggering the need for additional studies in dogs. The study was re-started in the same year, testing single ascending doses of treatments. Safety profiling in additional cohorts is ongoing [27].

2.2. Drug Repurposing for Human African Trypanosomiasis

Most drugs used to treat HAT have primarily been developed for that purpose rather than being repurposed from other indications. However, there are some approved or under investigation repositioned compounds.

Eflornithine (Fig. **1**), which was developed as an anticancer drug, has been successfully introduced for treating African trypanosomiasis [6]. The FDA approved it for the treatment of sleeping sickness in 1990. Additionally, Eflornithine is used for the treatment of *Pneumocystis carinii* pneumonia in AIDS patients and for treatment of anaplastic glioma [28].

Nifurtimox (Fig. **1**), identified in the late ´60s by Bayer in *in vitro* screening against *T. cruzi*, was marketed principally for use in the treatment of acute Chagas disease and back then it was the only and thus the front-line therapy for this indication. Nifurtimox has also shown activity against HAT, and the drug is effective against both the early and late stages of *T. b. gambiense* infection. Efficacy was first demonstrated in rat models and then in a limited clinical trial in four European patients. Subsequent trials with various Nifurtimox regimens revealed that cure rates were variable (30–80%), with toxicity accompanying higher doses and prolonged treatment [20]. Consequently, Nifurtimox was not approved as a monotherapy for second-stage HAT and is used primarily to treat those patients that are refractory to existing therapies. Despite the problems associated with its use, Nifurtimox has a significant place in HAT chemotherapy as combination therapy with Eflornithine [15].

The **combination of Eflornithine with Nifurtimox** was first evaluated for the treatment of late-stage *T. b. gambiense* infection in 2001 as part of a clinical trial comparing three drug combinations: Melarsoprol with Eflornithine, Melarsoprol with Nifurtimox, and Eflornithine with Nifurtimox [15]. Nifurtimox exerts its activity through induction of oxidative stress [29] while Eflornithine diminishes levels of trypanothione, a key metabolite to protect the parasite against oxidative stress; it was thus hypothesized that this drug combination might display synergic trypanocidal effects [30]. In 2009, the World Health Organization included the combination of Eflornithine and Nifurtimox in the Essential Medicine List. Later, in 2013, it was included in the Essential Medicine List for Children. Showing no differences in terms of effectivity compared to Eflornithine monotherapy, the combination results safer, cost-effective and requires shorter hospitalization.

Fexinidazole, (1-methyl-2-((p-(methylthio) - phenoxy) methyl)-5-nitroimidazole) was a long forgotten antiparasitic drug candidate until a few years ago (Fig. **1**). The drug had been in preclinical development in the 1970s and early 1980s as a broad-spectrum antimicrobial agent by Hoechst AG (now Sanofi-Aventis). However, Fexinidazole development was not pursued at the time. In the last decade, different studies have shown that Fexinidazole turned out to be an excellent candidate to cure HAT, including the advanced and fatal stage of the disease (stage 2) [31]. Fexinidazole and its metabolites require up to 48h exposure in order to induce maximal trypanocidal efficacy *in vitro.* Neither the parent drug nor its metabolites have displayed significant drug-interactions in terms of trypanocidal effect with either themselves or other known trypanocidal drugs in clinical use, thus precluding the possibility of combination therapies so far [32]. It is the first new drug candidate for the second stage of the disease that has reached clinical development in 30 years. Being an oral drug with the potential to be effective against both HAT stages caused by *T. b. gambiense* and *T. b. rhodesiense*, it could become the much needed breakthrough for HAT control by drastically simplifying case management [31].

Orlistat (also called Tetrahydrolipstatin) (Fig. **2**) is an FDA-approved anti-obesity drug targeting primarily the pancreatic and gastric lipases within the gastrointestinal tract. It has also displayed potentially interesting antitumoral and antibiotic effects. In 2006 Mackey *et al.* screened a library of 2160 FDA approved drugs and identified Orlistat as a possible hit against *T. brucei* [33]. Yang *et al.* found that Orlistat and some analogues possess potent trypanocidal activity, especially against the bloodstream form of *T. brucei*. Although its molecular mechanism has not been unveiled yet, Orlistat potency against *T. brucei* together with its well-characterized pharmacokinetic and safety profiles make it one of the most promising trypanocidal drug candidates for further development (IC_{50} 0.23 μM, *T. brucei* strain 427) [33-34].

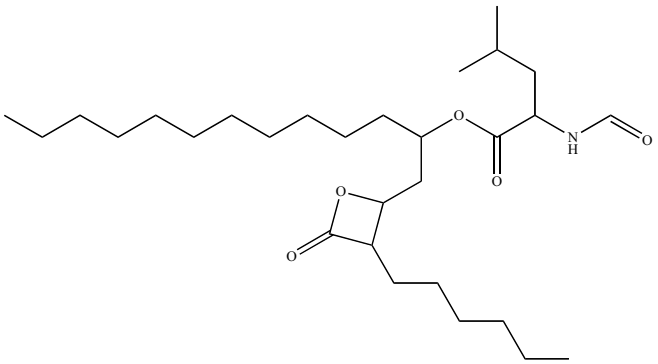


Fig. (2). Molecular structure of Orlistat.

Kinase inhibitors have come to the fore as one of the principal enzyme target classes in drug discovery for a wide variety of indications, including cancer, inflammation, diabetes, and CNS diseases. In particular, a number of tyrosine kinase inhibitors have been approved for clinical use [42]. *T. brucei* expresses more than 150 kinases [43], a fact that has driven several efforts to identify known kinase inhibitors that may be useful for the discovery of new therapies against HAT. A single Aurora-like kinase, TbAUK1, is responsible for promoting spindle assembly, chromosome segregation as well as cytokinesis in this parasite [44].

In 2009, Li *et al.* reported that **VX-680** (Fig. **3**), an Aurora kinase inhibitor originally targeted as an antitumoral

agent, also showed inhibitory effect on TbAUK1 with an IC_{50} value of 190 nM [45]. The drug arrested trypanosome cell proliferation in the G2/M phase, with an enlarged nucleus and two segregated kinetoplasts in each cell. The effect of VX-680 on trypanosome *in vitro* proliferation was investigated, finding an estimated IC_{50} of 10 μM.

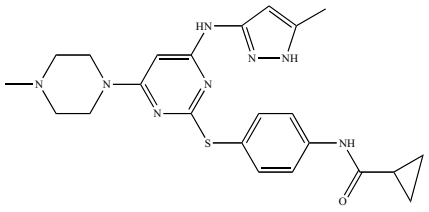


Fig. (3). Molecular structure of VX-680.

Also in 2009 it was reported that another human Aurora kinase antitumoral drug, **Hesperadin** (human Aurora B inhibitor), exhibits potent inhibitory effects on proliferation (IC_{50} = 50 nM on bloodstream forms) [46]. Hesperadin blocked nuclear division and cytokinesis, but not other processes of the cell cycle. Growth arrested cells accumulated multiple kinetoplasts, flagella and nucleoli. Later, Patel *et al.* designed and tested hesperadin analogues and identified non-toxic derivatives with inhibitory effects against *T. brucei*, *Plasmodium falciparum* and *Leishmania major* (counter-screen against the hepatic cancer cell line HepG2 was used as a general surrogate for host cell toxicity)[47]. Hesperadin molecular structure and the molecular structure of its more potent derivative against *T. brucei* are shown in Fig. **4A**.

Related to kinases pathway, in 2013, the same group evaluated the trypanocidal properties of a panel of **epidermal growth factor receptor (EGFR) inhibitors** provided by GlaxoSmithKline [42]. Despite lacking receptor tyrosine kinases, *T. brucei* has shown sensitivity to anticancer drugs Lapatinib and Canerinib [48] and other members of the same family with activity against EGFR [42]. Affinity chromatography and bioinformatics studies led to identification of five novel binding-proteins presumably involved in the effect of 4-anilinoquinazolines on *T. brucei* [48]. Subsequent design and synthesis of novel analogues allowed establishing structure–activity relationships for a 6-phenyl 4-anilinoquinazoline scaffold, culminating in a highly potent **Lapatinib analogue** against *T brucei brucei:* Lister 427 (Fig. **4B**). At cellular level, this analogue blocks duplication of the kinetoplast and arrests cytokinesis [42]. Although the compound has high calculated lipophilicity and molecular weight, Patel *et al.* observed modest effects in controlling parasitemia, with concomitant life extension of infected mice. Since pharmacokinetic studies suggest acceptable plasma levels in mice following oral dosing, it was hypothesized that the high binding to plasma proteins (99.6%) observed for this analogue might be the cause of the lower-than-expected effect on *in vivo* parasite loads. According to the authors, future efforts will focus on reduction of molecular size and lipophilicity of the analogues to improve the probability of CNS penetration [42].

In the same line, Ochiana *et al.* reported the repurposing of a human Aurora kinase inhibitor scaffold as specific inhibitor of trypanosomal Aurora kinase 1. Structure–activity relationship (SAR) analysis was carried on established human Aurora kinase inhibitors, focusing on decreasing the activity against the acute myelogenous leukemia cell line (MOLT-4) while maintaining the activity against *T. brucei rhodesiense* (Fig. **4C**). No animal studies have been reported to the moment [49].

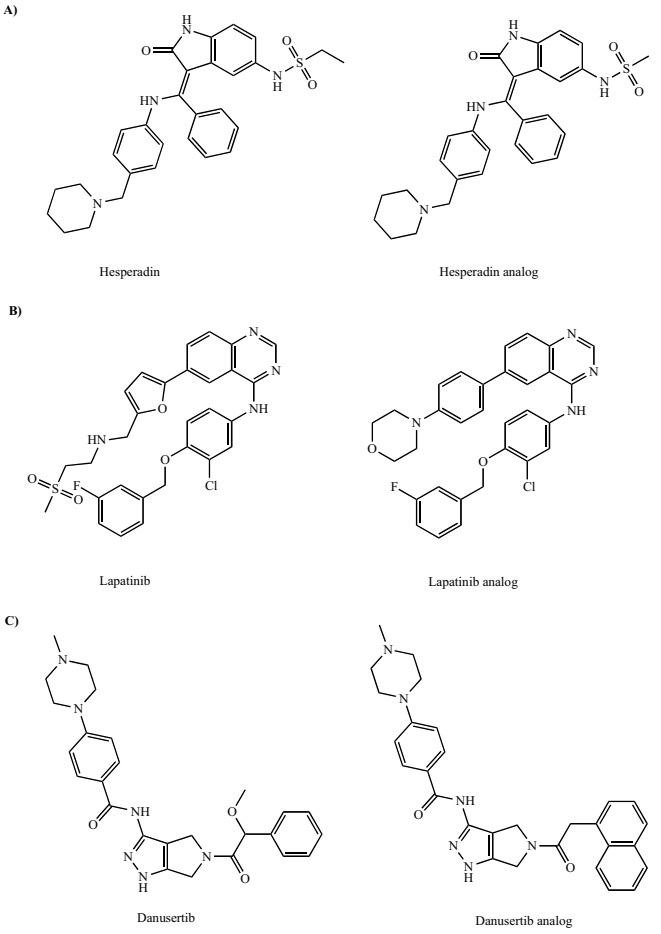


Fig. (4). Molecular structures of inhibitors of human kinases repurposed as potential treatments of *T. brucei,* along with potent and selective analogs.

More recently, Diaz and coworkers tested a wide set of human kinase inhibitor chemotypes to identify a diversity of starting points for HAT therapeutics development. A high-throughput screen (HTS) of 42,444 focused inhibitors from the GlaxoSmithKline screening collection was performed against parasite cell cultures and counter-screened against human hepatocarcinoma (HepG2) cells. 797 sub-micromolar inhibitors of *T. brucei* growth were identified, showing at least a 100-fold selectivity over HepG2 cells. 242 of these hit compounds displayed rapid inhibitory effect on cellular growth, and 137 showed rapid trypanocidal effects. A diversity of *in vitro* and *in silico* ADME-related properties (e.g. phospholipid binding, Chromlog D) were assessed. On the basis of the preceding studies, three compounds (Fig. **5**) were prioritized for *in vivo* pharmacokinetic studies and murine infection models of *T. brucei rhodesiense* was achieved with one of these compounds. Unfortunately this drug candidate is unlikely to penetrate into the CNS [50], thus presenting limited perspectives *per se* as HAT therapy.

Repurposing of **phosphodiesterase inhibitors** against trypanosomal phosphodiesterases represents an approach for the discovery of drugs for African sleeping sickness and Chagas disease [51]. Amata *et al.* synthesized analogues of Cilomilast, an orally active and selective human phosphodiesterase 4D (hPDE4) inhibitor originally developed by GlaxoSmithK for the treatment of respiratory disorders such as chronic obstructive pulmonary disease (COPD) [52]. SAR analysis led to the design and synthesis of highly selective inhibitors of TbrPDE4, which are shown in Fig. **6**.

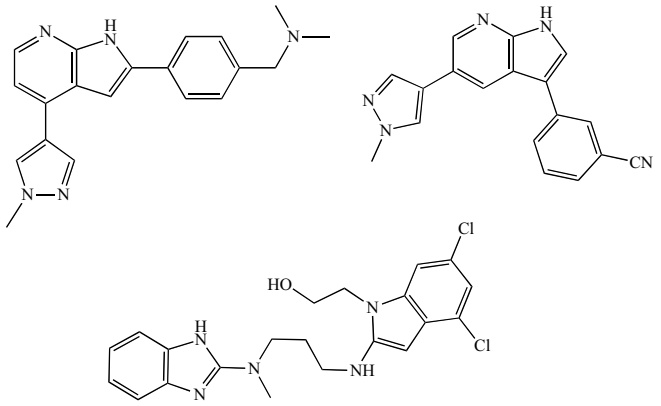


Fig. (5). Kinase inhibitors with antitrypanocidal effect found through HTS by Diaz *et al.*

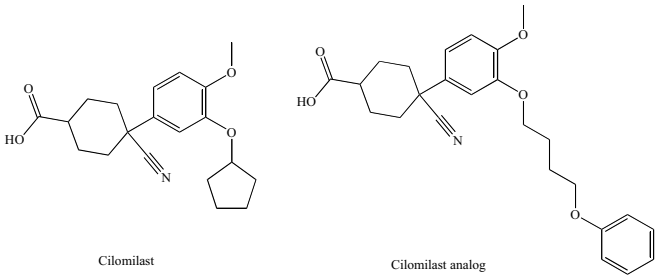


Fig. (6). Molecular structures of PDE4 inhibitors with inhibitory effect on *T. brucei.*

Bisphosphonates are nonhydrolyzable pyrophosphate (P-O-P) analogues in which the oxygen bridge has been replaced by a carbon (P-C-P). Several bisphosphonates are potent inhibitors of bone resorption and are in clinical use for the treatment and prevention of osteoporosis, Paget's disease, hypercalcemia caused by malignancy, and tumor metastases in bone. Their selective action is based on the binding of the bisphosphonate moiety to the bone mineral and the inhibition of the osteoclast's farnesyl pyrophosphate synthase (FPPS) [53]. This enzyme is responsible for the formation of farnesyl pyrophosphate (FPP), a compound used in protein prenylation and in the synthesis of dolichols, ubiquinones, heme *a*, and sterols.

A number of bisphosphonates, such as Risedronate, have recently been shown to have significant *in vivo* and /or *in vitro* activity against trypanosomatid and apicomplexan parasites [54-57]. Particularly, Martin and coworkers explored the *in vitro* activity of a series of bisphosphonates against the parasitic protozoa *T. brucei*, *T. cruzi*, *L. donovani*, *Toxoplasma gondii*, and *P. falciparum* [57]. The results show that nitrogen-containing bisphosphonates of the type used in bone resorption therapy have significant activity against parasites, with the aromatic species having in some cases nanomolar or low-micromolar IC_{50} activity values against parasite replication (e.g. o-risedronate, IC_{50} = 220 nM for *T. brucei* rhodesiense; risedronate, IC_{50} = 490 nM for *T. gondii*). On the other hand, Montalvetti and coworkers infected mice with *T. brucei* and tested different bisphosphonates. The most effective compound tested was Risedronate, which afforded a 60% cure (survival of 3/5 mice) at a dose of 2 x 5 mg/kg per day for 5 days, intraperitoneally. This demonstrates that bisphosphonates can suppress parasite proliferation *in vitro* as well as *in vivo* and that bisphosphonates may thus be useful lead compounds for the synthesis of new drugs effective against African sleeping sickness [54]. Thus, the FPPS of *T. brucei* (TbFPPS) turned an attractive target for drug development.

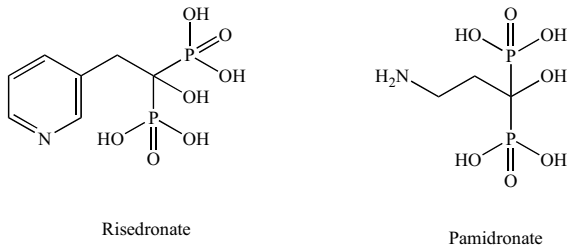


Fig. (7). Molecular structures of different bisphosphonates with inhibitory effect on *T. brucei, T. cruzi* and *Leishmania.*

3. CHAGAS DISEASE

Chagas disease or American trypanosomiasis is a tropical parasitic disease caused by the hemoflagellate protozoan *Trypanosoma cruzi*. The more frequent mode of transmission is through an insect vector commonly known as "kissing bug". Other transmission ways include congenital transmission, blood transfusion and organ transplant, contaminated foods and laboratory accidents [58]. Chagas disease involves two phases: i- an initial acute phase mainly characterized by mild nonspecific symptoms (fever, headache, muscle pain and others) or no symptoms at all and; ii- a chronic phase, which is asymptomatic in around 70% of the patients but includes life-threatening heart and digestive disorders in 30% and 10% of the chronically infected people, respectively. Though historically endemic to Latin America, Chagas disease is currently expanding to non-endemic countries and regions [59].

Along its life cycle, *T. cruzi* presents different cellular stages. Three of these forms, slender and broad trypomastigotes and amastigotes may be present in peripheral blood of an infected mammalian host. During a blood meal, the insect vector ingests the parasites that undergo differentiation in the bug midgut, from different stages to finally become epimastigotes. These epimastigotes migrate to the hindgut and attach to the epithelial intestinal cells prior to differentiating into the infectious metacyclic trypomastigote (process called "metacyclogenesis"). Later, they migrate to insect rectum to be excreted with feces, that can be inoculated in the vertebrate host. Metacyclic trypomastigotes can invade different cells, such as fibroblasts, macrophages, and epithelial cells, starting with the intracellular cycle of *T. cruzi.* After a transitory period in a vacuole, tryposmastigotes conclude their differentiation into amastigotes, the intracellu-

lar replicative form, in cytoplasm. Finally, amastigotes transform into trypomastigotes that are released to the extracellular space and may infect other cells or reach the bloodstream [14].

According to the most recent WHO estimates [58] there are presently around 6 to 7 million patients with Chagas disease. Treatment options are extremely limited, as discussed in the following sub-section.

3.1. Drugs and Treatment

Only two approved drug treatments are available, **Benznidazole** and **Nifurtimox** (Fig. **8**), and more than four decades have gone by since their discovery. Nifurtimox's action mechanism is not completely understood. Reduction of its nitro group to unstable anionic radicals that lead to formation of reactive oxygen species is the most probable effect, since *T. cruzi* lacks efficient detoxification mechanisms for such metabolites and is very sensitive to oxidative stress [60-61]. Additional molecular action mechanisms involving cytotoxic nitrile metabolites have also been reported [62-63]. Benznidazole mode of action involves covalent modification of biomolecules owing to reactive intermediates from nitroreduction [60, 64]. The main limitations of these drugs are their highly frequent side effects, which occur in up to 40% of the patients [58] and the presumably low cure rate in chronic stage (10-20%) [61, 65-66], though long-term follow-up clinical trials of Benznidazole in adults are currently concluding and will shed more light on the true efficacy in this stage of the disease [67].

The BENEFIT (BENznidazole Evaluation For Interrupting Trypanosomiasis) trial is the largest multicentre, randomized clinical trial, double-blind ever conducted on patients with *T. cruzi* infection and evidence of early Chagas cardiomyopathy. Patients were randomized to receive benznidazole (5 mg/kg per day) or matched placebo, for 60 days. BENEFIT has assessed the role of etiologic treatment with Benznidazole on a composite endpoint of: death, rescued cardiac arrest, sustained ventricular tachycardia, implant of a pacemaker or implantable cardiac defibrillator, heart failure, stroke or systemic embolism and heart transplant [68]. The BENEFIT program determined a number of outcomes related with parasitological and clinical characteristics in several countries. Differences in clinical manifestations between southern cone and more northern strains of *T. cruzi* have been suggested but are not clearly established. The results of this program have recently been published, providng valuable information about the role of benznidazole treatment in this phase and better understanding of the clinical progression of Chronic Chagas' cardiomyopathy [68].

Other national or international programs are also in course such as BERENICE (BEnznidazol and Triazol REsearch group for Nanomedicine and Innovation on Chagas disease - http://www.berenice-project.eu/index.php?option=com_content&view=article&id=15&Itemid=121&lang=en).

Other issues related to these medications include the length of the treatment regimens (60-day long for benznidazole, 90-120 day-long for Nifurtimox) [65, 69] and accessibility problems. Benznidazole is considered the first-line treatment, while Nifurtimox is reserved for those patients that do not tolerate the former [69-70].

The notorious biological, biochemical and genetic diversity of the T. cruzi strains (consensually organized in six discrete typing units, DTUs I-VI, by proximity in genetic baggage and by shearing common molecular markers) must be considered as another level of complexity in the search of effective treatments for Chagas disease [71]. While correlation of the clinical features of chronic Chagas disease with DTUs has not been really demonstrated, different strains have shown different response to drugs in *in vitro* or *in vivo* assays, explaining almost part of the failure of tested drugs in clinical trials where mixed infections occurred (eg. with azoles as Posaconazole and prodrug E1224, see next section). Given the importance of this point, the screening of new compounds should be done against a panel of strains of *T. cruzi*, prioritizing those that present effect on DTUs that are more often associated with Chagas infection in humans [72-73].

3.2. Drug Repurposing for Chagas Disease

The most promising new treatments for Chagas disease currently being investigated in clinical trials are, precisely, repurposed drugs. Among them, a number of azole antifungal drugs are maybe the most promising candidates [67].

Posaconazole (Fig. **8**) a structural analogue of Itraconazole, was approved by the US Food and Drug Administration (FDA) in September 2006 [74]. Posaconazole is a broad-spectrum triazole antifungal agent, it is approved for the prevention of invasive aspergillosis and candidiasis in addition to the treatment of oropharyngeal candidiasis [75]. In 1981, Docampo and coworkers pioneer work showed that the antifungal compounds Miconazole and Econazole inhibited ergosterol synthesis and proposed the use of azole compounds against *T. cruzi* [76]. Later in 1998 Urbina *et al.* first reported the *in vitro* and *in vivo* antiproliferative effects of Posaconazole on *T. cruzi* [77]. Alike fungi, *T. cruzi* depends on endogenous ergosterol and its derivatives [78] which are crucial for normal functioning of the parasite membranes, cell division, growth and development [79]. Moreover, inhibition of ergosterol biosynthetic pathway leads to cytotoxic accumulation of abnormal amounts of sterol precursors [78].

Posaconazole has demonstrated efficacy on several nitroderivative-resistant strains [80]. Back in 2012, it entered clinical trials in Argentina, Bolivia and Spain; the results of such studies showed that at the doses and treatment duration used, it is clearly inferior to the standard therapy, possibly due to low systemic bioavailability or treatment duration [67, 81,104]. Following that study, Merck started a second one to investigate the combination therapy of Posaconazole and Benznidazole, which was concluded in 2014; results are still awaiting publication [82]. It has been pointed out that even if these trials were successful, Posaconazole is highly expensive due to the low yielding and costly synthetic scheme, which threatens its widespread use in endemic countries [83]. It is interesting to mention in this context, however, that Posaconazole has been proved to be very effective in at least one recent case of compassionate treatment. A patient with systemic lupus erythematosus, after being immunosuppressed, developed an acute infection from a hidden Chagas

disease, and was successfully cured with Posaconazole [84]. Similarly, in a case of an Old World Cutaneous Leishmaniasis caused by *Leishmania infantum,* treatment with Posaconazole was successful [85].

Ravuconazole and E-1224 (a Ravuconazole prodrug) (Fig. **8**), other antifungal azoles, have also displayed potent *in vitro* activity against *T. cruzi.* Despite the Ravuconazole unfavorable pharmacokinetic profile in animals (characterized by very short elimination half-life), its pharmacokinetic parameters in humans prompted a proof-of-concept clinical trial of E-1224 [86]. E-1224 failed to develop sustained efficacy one year after the treatment in comparison with Benznidazole and presented some safety issues at high doses [84]. Further trials of E-1224 as a combination therapy with Benznidazole have been announced [67].

Other selective trypanosomal ergosterol biosynthesis inhibitors, **VNI** and **VFV** can cure acute and chronic experimental Chagas disease with 100% efficacy. Furthermore, VFV also suppresses visceral leishmaniasis by 89%, and displays low off-target activity, favorable pharmacokinetics and tissue distribution, being a promising new drug candidate for these diseases [87-88].

Fexinidazole (Fig. **8**), as has already been discussed in the HAT section of this article, is a nitroimidazol presently going through Phase II/III clinical trials for that disease. In 2012, a study showed comparative effects between Benznidazole and 3X higher doses of Fexinidazole on Benznidazole-susceptible CL *T. cruzi* strain, and on the partially resistant Y strain, while a superior effect of Fexinidazole was observed on Benznidazole-resistant VL-10 and Colombian strains in mice models of acute and chronic infection [89]. In the same study, Fexinidazole was also shown to reduce myocarditis in animals infected with VL-10 and Colombian strain. These results set the basis for the beginning of a Phase II proof-of-concept trial to determine the efficacy of the compound in adults with chronic indeterminate disease, which is being held in Bolivia.

Amiodarone (Fig. **9**), among the drugs repurposed as potential treatments for Chagas disease, has called much attention since it is commonly prescribed in patients with frequent complex arrhythmias derived from Chagas cardiomiophaty. This drug was first described as a potential antimicotic [90-91] before finding that it possesses a strong antiparasitic effect against *T. cruzi* [92]. Amiodarone is a class III antiarrhythmic agent that shares many characteristics of other elec-

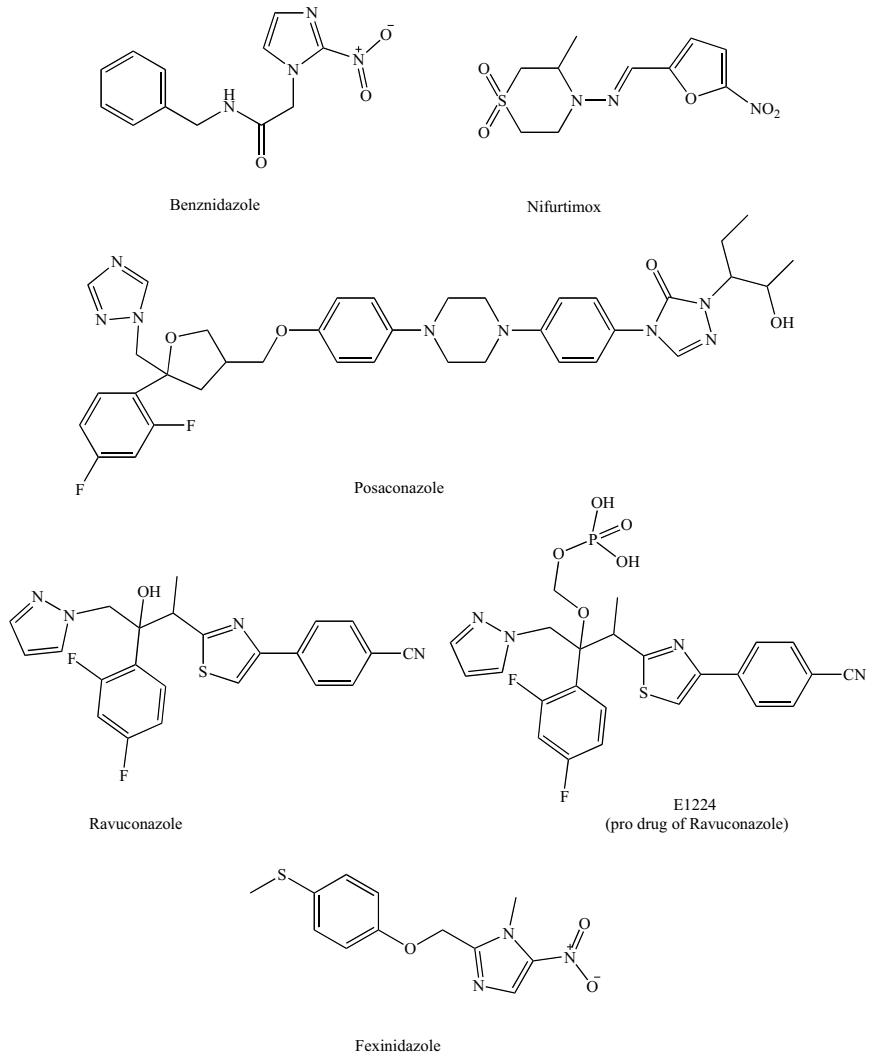


Fig. (8). Molecular structures of approved drugs and repositioned drug candidates against Chagas disease that have entered clinical trials.

trophysiological class of antiarrhythmic drugs, including inhibition of Na^+ and K^+ channels, L-type Ca^{2+} channels, the Na^+/Ca^{2+} exchanger, and non-competitive blockade of α and β adrenergic receptors [92]. Interestingly, some studies showed that patients with chagasic cardiomiopathy treated with Amiodarone had a more rapid and better recovery when compared with other patients treated with other class I and class IV antiarrhythmic agents.

Amiodarone treated patients usually exhibit a rapid increase in their ejection fraction and a decrease of their arrhythmogenic status with reduction on the frequency of their congestive heart failure exacerbation episodes [93-94]. These important changes represent the clinical translation of the underlying molecular mechanisms of Amiodarone on cardiac myocytes, which has proved to promote cardiac cell recovery with gap junction and cytoskeleton reassembly *in vitro* [95]; thus decreasing the rate of discharge and contractile performance. These facts were not easily explained by the electrophysiological properties of this drug alone, but suggested that other mode of action could be in play. In fact, it was demonstrated that Amiodarone was able to act directly on the parasite survival (both *in vitro* and *in vivo*), affecting the growth of *T. cruzi* extracellular epimastigotes and *T. cruzi* amastigotes, the clinically relevant stage of the parasite, without any evidences of damage of the host cell. The mechanism of action of Amiodarone was elucidated, showing that this drug directly disrupts the intracellular Ca^{2+} regulation of the parasite [92]. Such disruption has been postulated as a possible target of drug action, based in the differences among the mechanism of Ca^{2+} homeostasis described in human cells and those found in trypanosomes [96] and may be related to the trypanocidal effects of other calcium blockers from the dihydropyridine familiy, which are discussed later. Amiodarone induces a large and rapid increase in the intracellular Ca^{2+} concentration derived by Ca^{2+} release from intracellular organelles. This is the consequence of the collapse of the mitochondrial membrane potential (ΔΨ), and also of the alkalinization of the acidocalcisomes [92]. These curious and representative acidic intracellular compartments accumulate large amounts of Ca^{2+}, as well as polyphosphates and pyrophosphates, and are thus highly involved in the bioenergetics of trypanosomes [97]. It is worthwhile mentioning that pyrophosphate is considered an energetic coin alternative to ATP in trypanosomatids and other human parasites as *Plasmodium sp*. In fact, acidocalcisomes have been claimed as a rational target for the therapy of these parasitic diseases [96].

Besides its action disrupting the intracellular Ca^{2+} homoestasis of *T. cruzi*, Amiodarone is able to inhibit oxidosqualenecyclase, a key enzyme in the synthesis of ergosterol [98].

When used in combination, Amiodarone and Posaconazole showed a high synergistic action on host-cells infected with amastigotes, with a Fractional Inhibitory Concentration (FIC) index of 0.42 [92]. This is an interesting fact in favor of a possible combinatory use of these drugs in human, each at a lower concentration, with the beneficial implication of producing fewer side effects.

As a very promising result, Amiodarone and Itraconazole (the azol which Posaconazole is derived from), have been used in combination as a compassionate treatment, in at least

Table 2. Approved and experimental drugs at clinical stage for the treatment of Chagas disease.

Drugs	Stage disease	*In vitro* trypanocidal activities (IC_{50})	Adverse Effect	References
Nifurtimox	Acute phase (for those patients that do not tolerate Benznidazole)	3.50±0.58 μM(strain Y – epimastigote)	Anorexia, weight loss, psychic disorders, irritability, insomnia, nausea, diarrhea	[60-63, 69-70, 101-102]
Benznidazole	Considered the first-line treatment for Acute phase and in newborns and children (to 16 years old) Chronic phase – under test	20.35±3.04 μM (strain Y – epimastigote)	Rashes, peripheral neuropathy, hypersensitivity syndromes with fever, lymphadenopathy, exfoliative dermatitis, anorexia, nausea, vomiting, weight loss and insomnia.	[[64, 67, 70, 102-103]
Posaconazole	All stages of the disease	25 nM (epimastigotes strain Y)	Drug interactions related to CYP3A4 inhibition. Caution must be taken when co-administered with other CYP3A4 substrates. Care must be taken when administered to a patient with arrhythmic disorders or taking pro-arrhythmic drugs.	[104-107]
Ravuconazole and E-1224	All stages of the disease	0.1 nM (*T. cruzi* amastigotes)	Not informed	[108-109]
Fexinidazole (o)	All stages of the disease	29 μM (epimiastigotes strain Y)	Headache and vomiting of acceptable intensity	[67, 110]

one human case, resulting in the parasitological cure of the patient [93]. However, since Amiodarone presents some secondary adverse effects as pulmonary fibrosis and thyroid toxicity because of the presence of iodine in its structure, an effort to synthesize an analog with less undesirable side effects were made. Dronedarone (Fig. **9**) arose as a possible improvement, lacking iodine and with a less hydrophobic character, to avoid the excessive tissue accumulation typical of Amiodarone. Thus, the effect of this new derivative was studied on *T. cruzi* [99] as well as in *L. mexicana* [100]. This drug shows the same essential facts of its precursor but, interestingly, with an improved activity, since its IC_{50} is lower than Amiodarone's and its effect on the parasite mitochondrial potential and on acidocalcisome alcalinization is faster [100]. It is worthwhile mentioning that the IC_{50} of Dronedarone on *L. mexicana* amastigotes-infected macrophages is 3 orders of magnitude lower than that obtained on *T. cruzi* amastigotes (0.65 nM versus 0.75 μM) [100] (Table **2**).

Similar to Amiodarone, Dronedarone inhibits parasite oxidosqualene cyclase. Taken together, all these results suggest the possibility of repurposing Dronedarone as a treatment for Chagas disease and Leishmaniasis.

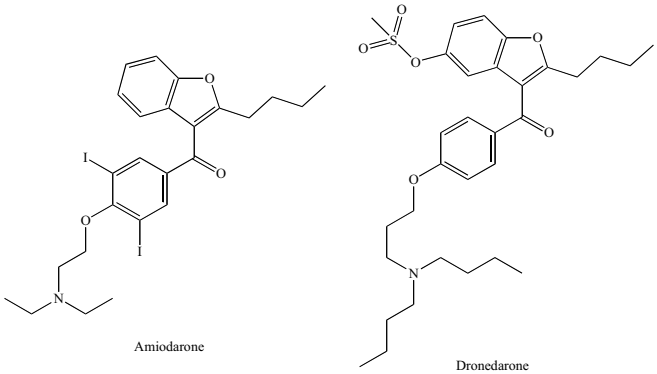


Fig. (9). Structures of Amiodarone and Dronedarone.

SQ109, (Fig. **10**) in advanced medical trials against resistant forms of **Mycobacterium tuberculosis**, also shows activity against other bacteria, fungi, and a malaria parasite. First reported to act by inhibiting the transporter MmpL3, involved in cell wall biosynthesis, it was later reported that it also acts on the menaquinone biosynthesis, on electron transport, inhibiting respiration and ATP biosynthesis, and finally as an uncoupler, driving the collapse of the mitochondrial membrane potential [111]. Very recently it was reported that SQ109 shows a potent anti-*T. cruzi* activity, acting on all forms of the parasite, trypomastigotes, epimastigotes, and even in the clinically relevant form, intracellular amastigotes, causing major ultrastructural changes in all forms. SQ109 also acts synergistically with Posaconazole on intracellular amastigotes with a FIC of 0.48. Moreover, it induces the rapid alkalinization of the parasite´s acidocalcisomes [112], being thus mechanistically related to Amiodarone. Docking approaches also showed that SQ109 is able to bind *T. cruzi* squalene synthase, an important enzyme involved in the ergosterol synthesis of this parasite [112]. These results indicate that SQ109 also could be considered as a potential drug against Chagas disease.

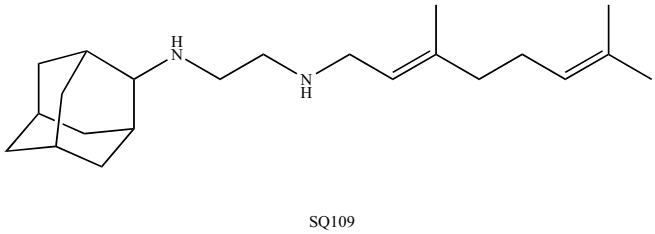


Fig. (10). Molecular structure of SQ109.

Bisphosphonates, suchs as the **N-alkil substituted,** used in the treatment of bone disorders, have displayed selective *in vitro* and *in vivo* inhibitory effects on *T. cruzi* [113], by the inhibition of the *Tc*FPPS, as previously mentioned (in 2.2 Drug repurposing for Human African trypanosomiasis section) [57]. **Risendronate** is the biphosphonate that has exhibited the most potent effect found on *T. cruzi* [57]. At doses of 1 mg/kg per day it induces reductions in parasitaemia above 90% in mice acute models of infection and no relapse after discontinuation of the treatment [114]. **Lipophilic bisphosphonates** affect *T. cruzi* proliferation by inhibiting endogenous sterol biosynthesis [115]. In *in vitro* assays, these compounds have proven to be potent inhibitors against the intracellular form of *T. cruzi* exhibiting IC_{50} values at the low micromolar to nanomolar range [116-118].

Other compounds, as aryloxyethyl thiocyanate derivatives (by inhibiting squalene synthase) [119], or those structurally related to 4-phenoxyphenoxyethyl thiocyanate with their terminal aromatic ring modified [120] have also shown promising results as good candidates to new therapies for Chagas disease.

In a very recent article, Planer et al reported the test of 2,000 biologically active compounds from a **Spectrum library** including about 700 FDA approved drugs, on mammalian stage *T. cruzi* [121]. Most of the identified active compounds (except the antifungal azoles) showed activity in the low micromolar or high nanomolar range (among them, antidepressant Fluoxetine and the antihistamine Clemastine) but they were inefficient to lower parasitemia in a murine model of infection. Therefore, the authors decided to test combinations of active compounds in search of synergistic and additive effects. Isobologram studies revealed 8 synergistic drug pairs, among which the **combinations of Posaconazole plus Clemastine and calcium blocker Amlodipine** provided the best outcome.

Other very frequently selected target for drug repurposing is **cruzipain,** the major cysteine-protease of *T. cruzi*, due to its participation in parasite invasion and reproduction [122]. Many cysteine protease inhibitors have been studied, one of this, the **vinylsulfone K777**, has been proven effective in animal models of Chagas disease, but drug development progress was halted in 2005 due to hepatotoxicity [123]. Other approved drugs chemically related to K777 are being studied nowadays.

Bellera *et al.* have recently developed **virtual screening campaigns for computer-assisted drug repurposing** to identify new cruzipain inhibitors as potential treatments against Chagas disease [124]. After screening DrugBank and Merck Index 13th libraries, the authors applied different selection criteria related to the steady-state plasma concentra-

tions achieved for the original therapeutic indication and the most frequent side-effects linked to the original therapeutic use to decide which candidates deserved being moved to animal models. **Benidipine** was selected on the basis of its cardioprotective properties (which might pose and added value to patients with Chagas) and its mild side-effect, while **Clofazimine** was selected owing to its acceptable safety profile and the similarity between the steady state concentration achieved when used for the original indication and the concentrations that showed activity on the parasite in cellular studies. Both candidates reduced the parasitemia in a murine acute model of *T. cruzi* infection, though Benznidazole 100 mg/kg/day outperformed Benidipine and Clofazimine. The authors highlighted, however that 5 to 10 times lower doses of the candidates were used, in comparison to the Benznidazole dose. Moreover, both repositioned candidates reduced the number of *T. cruzi* nests quantified by conventional microscopy of cardiac tissues from each treated animal.

4. LEISHMANIASIS

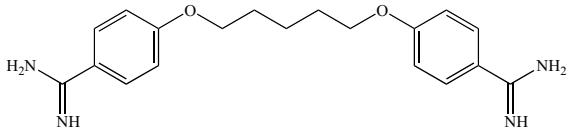
Leishmaniases are a complex group of diseases caused by more than 20 different species of the genus Leishmania. Leishmania parasites exist as exoflagellated promastigotes in the female phlebotomine sandfly vector and as amastigotes in their mammalian hosts. The promastigotes are injected into the mammalian host during the vector´s blood meal. Then, they are phagocytized by macrophages, dendritic cells and/or neutrophils attracted to the biting site in the skin. Once inside the phagosome, promastigotes differentiate into amastigotes and multiply in phagolysosome by simple division until bursting the host cell. In the mammalian host, these protozoa are obligate intracellular parasites of macrophage-dendritic cell lineages.

The disease mainly affects low-income people in Africa, Asia and Latin America, and is associated with malnutrition, population displacement, poor housing, weak immune system and lack of resources. There are four different clinical forms of Leishmaniasis: i- Cutaneous Leishmaniasis (CL), characterized by skin ulcers usually form on exposed areas, such as the face, arms and legs; ii- Mucocutaneous Leishmaniasis (MCL), with lesions that can partially or totally destroy the mucous membranes of the nose, mouth and throat cavities; iii- Visceral Leishmaniasis (VL) also known as kala-azar or black fever in the Indian sub-continent, which is the most severe form of the disease, fatal if untreated, that is characterized by high fever, substantial weight loss, swelling of the spleen and liver, and anaemia; and iv- Post-Kala-azar Dermal Leishmaniasis (PKDL). Over 90% of VL cases occur in six countries: Bangladesh, Brazil, Ethiopia, India, South Sudan and Sudan. CL cases occur in Afghanistan, Algeria, Brazil, Colombia, the Islamic Republic of Iran, Pakistan, Peru, Saudi Arabia and the Syrian Arab Republic. Finally almost 90% of MCL cases occurs in Bolivia, Brazil and Peru [125]. Factors determining the kind of clinical manifestation depend upon the infecting species and host factors, such as general health, genetic and immune constitution. All have different immunopathology and cause varying degrees of mortality and morbidity.

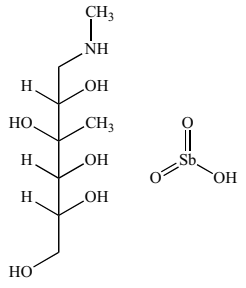
Similar to the other protozoan causing diseases, it is very difficult to arrive to a single drug capable of treating Leishmaniasis in every therapeutic scenario, due to the great variability of strains and clinical manifestations. First-line antileishmaniasis medicines include Sodium stibogluconate or Meglumineantimoniate. Nevertheless, there is an urgent necessity for new drugs for Leishmaniasis treatment due to the increased number of therapeutic failure caused by antimonial-resistant *Leishmania spp* strains (see below).

4.1. Drugs and Treatment

Pentavalent antimonials, as **Sodium stibogluconate** (Pentostam™, GSK) and **Meglumine antimoniate** (Glucantime, Aventis) are the classical treatments for Leishmaniasis (Fig. **11**). These compounds seem to have a broad mechanism of action. Data suggest pentavalent antimony (SbV) enters the host cells, crosses the phagolysosomal membrane and is converted into trivalent antimony (SbIII). Then, SbIII acts against amastigotes by compromising the cells thiol redox potential by inducing efflux of intracellular thiols and consequently inhibiting trypanothione reductase (TR) [126]. Antimonials also act at the DNA level, inducing DNA damage *in vivo* [127], and inhibiting DNA topoisomerase I [128]. Despite its documented toxicities, antimonials were the first-line therapy against VL. However, parasite resistance greatly reduces the efficacy of conventional medications [129]. In the last 15 years, clinical misuse of these medications has enabled the development of generalized resistance to these agents in Bihar, India, where half of the global VL cases occur [130]. Besides treatment failure, antimonials require up to 28 days parenteral administration [130-133].



Stibogluconate



Meglumine antimoniate

Fig. (11). Pentavalent antimonials for classical treatment of Leishmaniasis.

4.2. Drug Repurposing for Leishmaniasis

In the last decade, the number of approved or potential therapies against Leishmaniasis have arisen via drug repurposing; two of them, Paromomycin and liposomal Amphotericin B (see both below), were included in the Model List of Essential Medicines (17th edition) published by WHO in 2011 [134]. The following text resume the characteristics of approved or experimental repositioned drugs, classified accordingly to the original action (antifungals, antibiotics, anti-

tumorals, etc). Table **3** summarizes classical and repositioned drugs for the treatment of Leishmaniasis.

Amphotericin B (AmBD) is an antifungal compound used in the treatment of Aspergillosis, Cryptococcosis, Blastomycosis, Systemic candidiasis, Histoplasmosis and Zygomycosis [135] (Fig. **12**). It was first licensed as an intravenous therapy infusion in 1959 against life threatening fungal infections and then used in India for the treatment of antimonial-resistant VL. AmBD binds to ergosterol precursors interrupting parasite cell wall synthesis. AmBD also binds ergosterol in the membrane forming complexes that open pores which alter the ion balance and lead to cell death [78].

Although this drug has an excellent cure rate, it is highly toxic and needs to be administered by slow intravenous infusion; otherwise, patients can suffer adverse reactions to the infusion such as fever, chills, and thrombophlebitis. More severe life threatening side-effects including nephrotoxicity and myocarditis can also occur. The administration route and

Table 3. Approved and experimental drugs (at clinical stage) for the treatment of Leishmaniasis

Drugs	Stage disease	Doses and /or i*n vitro* leishmanicidal activities (IC_{50})	Adverse Effect	References
Pentavalentantimonials (IV, IM, IL)	Visceral Leishmaniasis kalaazar) and Cutaneous Leishmaniasis	28 days or more 20 mg/Kg/day, during	High cardiotoxicity. pancreatitis, nephrotoxicity, hepatotoxicity High treatment failure (up to 65% in major epidemic areas).	[130]
Amphotericin B, AmBD (IV) and Liposomal amphotericin B, AmBisome (IV)	Visceral Leishmaniasis	30 days 1 mg/kg (15 mg/kg total dose) for AmBD and 5–20 mg/kg total dose over 10–20 days for AmBisome	High nephrotoxicity, also myocarditis and death for AmBD and minimal side effects (rigors and chills) for AmBisome	[196]
Paromomycin sulfate (IV,IM, topic)	Visceral and Cutaneous Leishmaniasis	21 days 15 mg/kg/day	Gastrointestinal symptoms including nausea, vomiting, diarrhea and abdominal discomfort. Nephrotoxicity and ototoxicity are rarely produced.	[149]
Azithromycin	*Leishmania amazonensis, Leishmania braziliensis, and Leishmania chagasi* Cutaneous Leishmaniasis	IC(50) of 27 uM (*L. amazonensis)* 2.7 uM (*L. brazilienzis)* and 7.8 uM (*L. chagasi*).	Safe and well tolerated, diarrhea are the most frequent side effect	[141, 197]
Miltefosine (PO)	Visceral and Cutaneous Leishmaniasis	28 days 1.5–2.5 mg/day 1 uM (amastigotes)	Gastrointestinal symptoms, nephrotoxicity, hepatotoxicity, teratogenicity.	[151]
Imiquimod (topic)	Cutaneous Leishmaniasis	5% cream (applied over lesions 2 or 3 times/day) and combined with oral treatments (Glucantime, Miltefosine).	Well tolerated, mild to moderate erythema, edema, and sensations of burning.	[161]
Sunitinib, sorafenib and lapatinib	*L. donovani, and L. major, L. amazonensis and L. mexicana* (sorafenib)	50 mg/kg (PO) in mice/ 1.1, 3.7 and 2.5 uM (amastigotes)	Not available	[163]
Mianserin	*L. donovani*	21 μM (promastigotes) 46 μM (amastigotes)	Not available	[169]
Ketanserin	*L. donovani*	37 μM (promastigotes) 28 μM (amastigotes)	Not available	[170]
Sitamaquine (PO)	Visceral Leishmaniasis	1.5 - 3 mg/mg/day	Vomiting, abdominal pains and, headache, cyanosis and methemoglobinemia (in individuals with glucose-6-phosphate deshydrogenase (G6PD) deficiency) nephrotoxicity (in doses > 2,5 mg/mg/day)	[173]

toxicity of AmBD impose monitoring and hospitalization, which increase the cost of this therapy. New formulations, including liposomal AmBD (AmBisome; Gilead Sciences), amphotericin B lipid complex (ABLC; Abelcet, Enzon Pharmaceuticals), and amphotericin B cholesterol dispersion (ABCD; Amphotec™, Inter- Mune Corp.) which encapsulate AmBD within lipid-based advanced drug delivery systems, have been produced to overcome the preceding issues. These lipid-associated formulations of AmBD display reduced toxicity and increased plasma half-life. In 1997 AmBisome became the first FDA-approved drug for Leishmaniasis [136]. However, they are expensive therapeutics [8, 136-137]. Liposomal preparations are particularly beneficial in patients with renal impairment (a condition that precludes the use of amphotericin B deoxycholate) and those with febrile neutropenia [138].

Itraconazole and Posaconazole, azoles with antifungal effect (Fig. **12**), have been confirmed as effective compounds against *Leishmania amazonensis*, showing antiproliferative, physiological and ultrastructural effects. They act by inhibiting sterol C14a-demethylase (CYP51). As previously stated in the section dedicated to Chagas disease, inhibition of ergosterol biosynthesis is increasingly recognized as a promising target for the development of new chemotherapeutic agents.

Physiological studies revealed that both Itraconazole and Posaconazole induced a collapse of the mitochondrial membrane potential (DYm), which was consistent with ultrastructural alterations in the mitochondrion [139].

In vitro antileishmanial effects of Clotrimazole, Econazole and Bifonazole, all drugs derived from the antifungal Imidazole, have been reported and linked to regulation of reactive oxygen species (ROS) of the parasites [140].

Azithromycin, an azalide antibiotic closely related to the macrolides clarithromycin and erythromycin (Fig. **13**), is a non-expensive, largely commercially available drug widely used for the treatment of bacterial infections. Azithromycin has demonstrated activity against intracellular microorganisms as *Toxoplasma gondii* and *Plasmodium falciparum* [141], because it is highly concentrated within different phagocytic cells, especially macrophages. Azithromycin has also shown *in vitro* and *in vivo* activity against different species of Leishmania, as *L. (Leishmania) amazonensis, L. (Viannia) braziliensis, and L. (Leishmania) chagasi* [142-143].

Paromomycin is an antibiotic used to treat acute and chronic intestinal amebiasis and adjunctive management of hepatic coma, by suppressing the growth of intestinal bacteria that make ammonia (Fig. **13**). First isolated in the 1950s [144], Paromomycin is an aminoglycoside–aminocyclitol antibiotic of broad-spectrum effective against gram-positive and gram-negative bacteria. Paromomycin impairs the mitochondrial membrane potential, inhibits protein synthesis, and leads to respiratory dysfunction. It also alters membrane fluidity and lipid metabolism [145]. It is also effective against cestodes and other protozoa, including *Giardia lambdia* and *Entamoeba histolytica* [144]. The antileishmanial activities of Paromomycin were established in the 1960s [146-148]. Later it was used for parenteral treatment of VL and then, for CL [149]. More recently, Paromomycin has been combined with Gentamicin in an antibiotic preparation called "Walter Reed (WR) 279,396" for the treatment of CL; it has been tested as topical cream in clinical trials, for USA military health care in Southwest Central Asia/Middle East destinations, in Panama and in Tunisia [149-150].

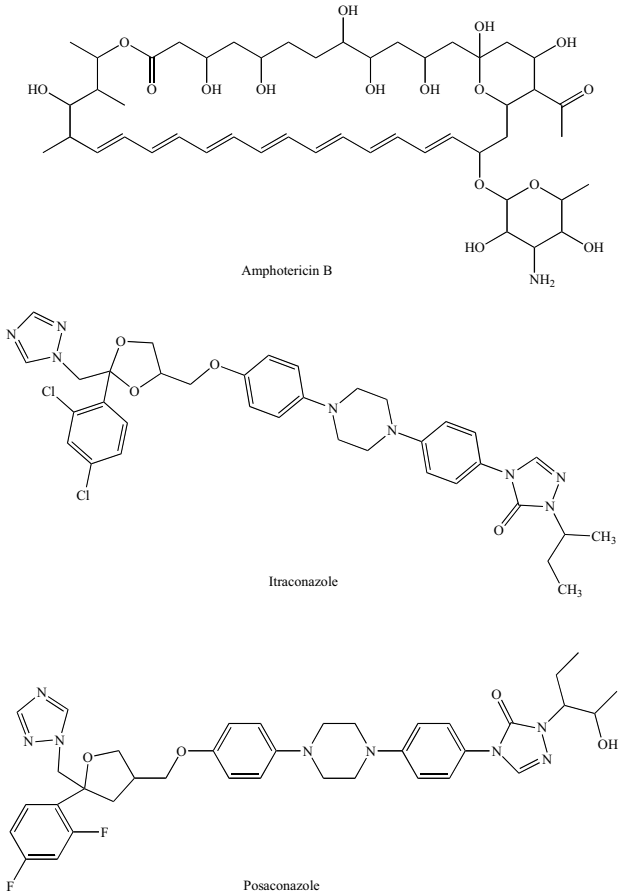


Fig. (12). Antifungals proposed as new therapies for Leishmaniasis

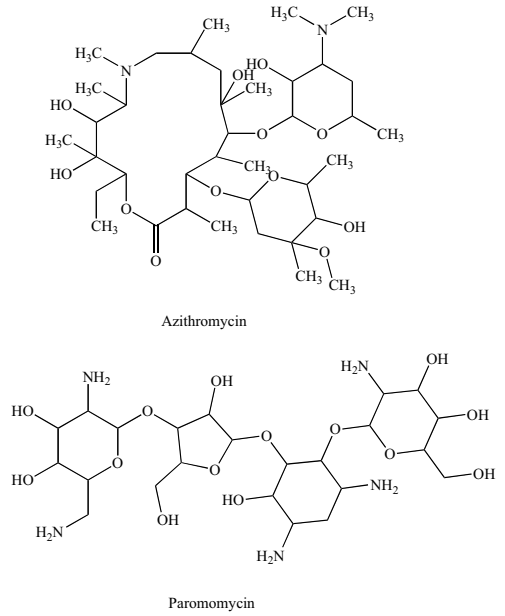


Fig. (13). Antibiotics repositioned as potential therapies against Leishmaniasis

Miltefosine (Impavido®) (Fig. **14**), used to treat skin metastases of breast cancers, was repurposed to VL [150-151] being the only drug registered for oral treatment of Leishmaniasis. The antitrypanosomal and antineoplastic effects of Miltefosine and related alkylphosphocholine drugs were simultaneously, but independently, discovered back in the 1980s [151-152]. Priority was given, however, to the development of miltefosine for local treatment of cutaneous metastases of breast cancer, with a topical formulation (Miltex, Baxter, UK) being approved for this indication [150]. While this compound was also studied for its potential use against other types of tumors, it was ultimately discontinued due to dose-limiting gastrointestinal side effects [151]. These anti-cancer studies did however lead to Miltefosine being reinvestigated for Leishmaniasis and, after several clinical trials, to its approval for oral treatment of Leishmaniasis. Today Miltefosine can be used to treat all clinical Leishmaniasis (Berman, 2008); however, it is associated with common gastrointestinal side effects. It is also limited by its relatively high cost [153], teratogenicity and growing concerns in relation to increased tolerance in clinical isolates (Prajapati *et al.*, 2012). The mechanism of action of Miltefosine has been linked to impaired lipid metabolism [154] and induction of parasite apoptosis [155]. Miltefosine was also shown to act at the host cell level stimulating the production of inducible nitric oxide synthetase 2 (iNOS2) that catalyzes the generation of nitric oxide (NO) to kill the parasite within macrophages [156].

An effective combination therapy of Miltefosine with AmBD or Paromomycin could be helpful to treat antimony-resistant infections in India [157]. It was also demonstrated that the combination of Miltefosine and Amiodarone produced the parasitological cure of cutaneous Leishmaniasis determined by PCR, in 90 % of mice infected with *L. mexicana* [158]. When the mice were treated with either drug alone, albeit the lesion disappears after 21 days of treatment, in both cases there was a relapse observed by the reappearance of the lesion after 48 days post-infection. The isobologram of Miltefosine and Amiodarone indicates a Fractional Inhibitory Concentration (FIC) of 0.48 [158].

Imiquimod (Aldara®; 3M Pharmaceuticals) is a novel synthetic immune response-activating compound, with a potent antiviral and antitumor effect in animal models (Fig. **14**). It has been approved by the FDA for cervical warts and, lately, it has been effective in activating macrophage killing of Leishmania species by releasing of nitric oxide [159].

Imiquimod increase the efficacy of Glucantime in a footpad infection model of BALB/c mice infected with *L. major* [160]. This combination was also used as rescue treatment in 12 patients with Peruvian CL who had previously not responded to Glucantime alone. This immunomodulator showed to be capable of decreasing the time to cure, potentially increasing the cure rate, in Andean CL [161-162].

Inhibitors of Protein kinase, used in the treatment of human cancer, have shown antileishmanial action. Sunitinib, Sorafenib and Lapatinib (Fig. **14**) were identified as active drug candidates against *L. donovani* amastigotes in cultured murine macrophages with IC_{50} values of 1.1, 3.7 and 2.5 μM, respectively, which is similar to the potency of Miltefosine (IC_{50} = 1.0 μM). They show no toxicity on mammalian cells. In addition, some of the protein kinase inhibitors were active against *L. donovani* in the BALB/c mouse model of infection; dosing on days 7–11 with a 50 mg/kg oral dose of Sunitinib, Lapatinib or Sorafenib reduced liver amastigote burden by 41%, 36% and 30%, respectively, compared with untreated control mice. Although less efficacious, Sorafenib was also active *in vitro* against intracellular amastigotes of the cutaneous disease-causing species *Leishmania amazonensis*, *L. major* and *L. mexicana* [163].

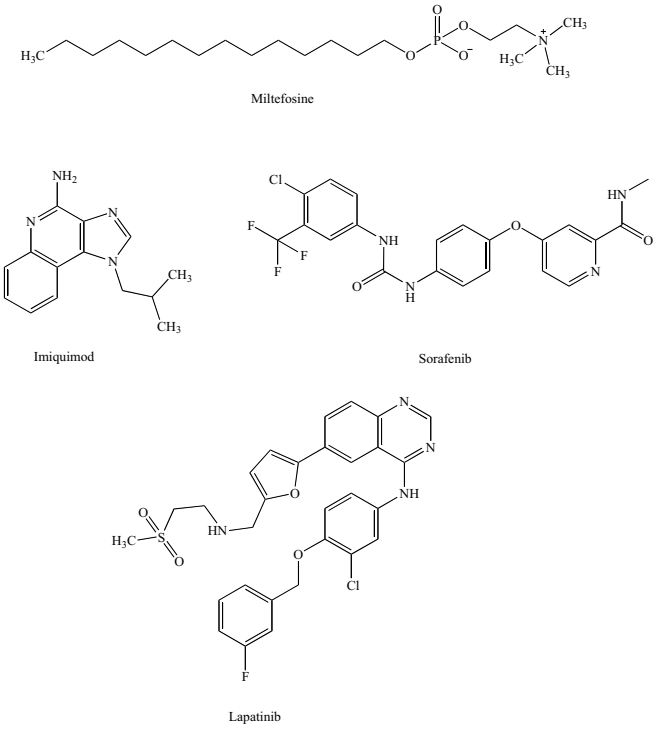


Fig. (14). Repositioned antitumor drugs as potential therapies against Leishmaniasis.

Bisphosphonates, as mentioned before, are potent protozoan parasite inhibitors. A number of bisphosphonates, such as Risedronate, Pamidronate, 2-phenyl-1hydroxyethane-1,1-bisphosohonate, 1- hydroxyhexane-1,1bisphosphonae, have recently been shown to have significant activity against *Leishmania donovani*, inhibiting the *in vitro* proliferation [54-55, 57]. The bisphosphonate Pamidronate (Aredia; Novartis) at intraperitoneal dose of 10 mg/kg/day for 5 days produces a radical cure of cutaneous leishmaniasis in Balb/c mice, as evidenced by long-term disappearance of lesions concomitantly to disappearance of amastigotes in lesion sites [164].

Mianserin, in its hydrochloride form, is classified as a noradrenergic and specific serotoninergic antidepressant (NaSSA) with a tetracyclic structure (Fig. **15**). It was at first used for the treatment of depressive illness and depression associated with anxiety. Its antidepressant effect is mainly attributed to presynaptic a2-adrenoreceptor blocking activity and to serotonin receptor antagonism [165]. Furthermore, Mianserin strongly blocks post-synaptic 5-HT2 receptors, while it weakly blocks post synaptic 5-HT1 and 5-HT3 receptors [166]. A number of studies have indicated that Mianserin differs from tricyclic antidepressants (TCAs) not only chemically but also in its pharmacological profile [167]. Mi-

anserin was originally designed as an anti-allergic drug and later as antidepressant after its effects in CNS activity were detected by computarized electroencephalographic studies in volunteers [168].

More recently, Mianserin was found to inhibit both the promastigote and amastigote forms of the *Leishmania* spp. in a dose dependent manner. The IC_{50} values for promastigotes and amastigotes were 21 μM and 46 μM respectively while is above of 100 μM for THP-1 differentiated macrophages exhibiting selective action on parasites. It has been reported that Mianserin's antileishmanial effect is linked to depletion of ergosterol levels by competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR, the rate-limiting enzyme of the ergosterol biosynthetic pathway) [169].

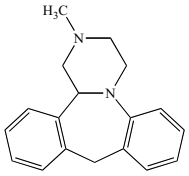


Fig. (15). The noradrenergic and specific serotoninergic antidepressant Mianserin, repositioned for Leishmaniasis therapy.

Ketanserin, a tricyclic serotonin S2-receptor antagonist used as an antihypertensive agent, has also shown an antileishmanial action (Fig. **16**). It was found lethal, in a dose-dependent manner, to both *L. donovani* promastigotes (with an IC_{50} value of 37μM) and intracellular amastigotes (IC_{50} = 28μM), with no apparent toxicity to the host cells. The effect was exerted by inhibition of *L. donovani* 3-hydroxy-3-methylglutaryl coenzyme A reductase [170].

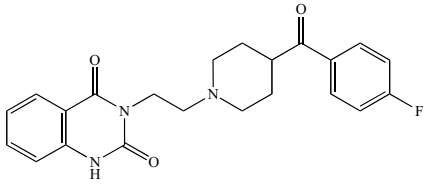
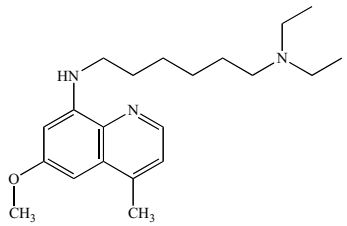


Fig. (16). Ketanserin, an antihypertensive agent, with probed antileishmanial action.

Sitamaquine is an 8-aminoquinoline which was originally described as an antimalarial drug [171] and later suggested as potential therapy against *T. cruzi* [172]. Some years ago, an oral Sitamaquine treatment was developed for VL (Fig. **17**). An advantage of Sitamaquine seems to be its short elimination half-life, which prevents a rapid resistance emergence. However, the selection of a sitamaquine-resistant clone of *L. donovani* in laboratory and some adverse effects such as methemoglobinemia and nephrotoxicity should be considered for a further development decision [173].

Similarly to the previous discussion on the effect of dihydropyridines against *T. cruzi*, **various VGCC antagonists** have been reported to inhibit the growth of different species of Leishmania. Among them, Nimodipine, a dihydropyridine-derivative, has been reported to possess antileishmanial activity, inducing ultrastructural alterations in *L. chagasi* [174], while Amlodipine and Lacidipine, common hypertensive drugs, both showed activity against VL on infected mice [175]. Also, Amlodipine showed an antiparasitic effect on VL alone and in combination with Pentamidine and other drugs. In this study, the combination of Nimodipine and Glucantime was the most promising in amastigotes [176]. On the other hand, both Nifedipine and the phenylalkylamine Verapamil, also a potent specific VGCC antagonist blocked the binding of *L. donovani* amastigotes to macrophages [177], supporting the notion of an essential role of Ca^{2+} during the process of macrophage infection [96]. All the above results point to the presence of an L-type VGCC analog in *Leishmania* parasites. In fact, a sphingosine-sensitive Ca^{2+} channel was recently described to be present in the plasma membrane of *L. mexicana*, with characteristics which closely resemble the *L*–type VGCC present in humans, as the blockade of Ca^{2+} entry by Nifedipine and Verapamil [178]. Two genes potentially coding for an *L*-type VGCC were identified in the genome of these parasites [178]. Concerning this point, it has been demonstrated that Miltefosine is able to increase the intracellular Ca^{2+} concentration of promastigotes of *L. mexicana*, by inducing the opening of a plasma membrane Ca^{2+} channel [158]. Interestingly, we have recently observed that Miltefosine actually acts on the just mentioned sphingosine-sensitive Ca^{2+} channel, and its action is blocked by *L*-type VGCC antagonists (*G. Benaim, unpublished results*).



Sitamaquine

Fig. (17). Sitamaquine, a putative antileishmanial drug candidate.

Pentamidine, **Ketoconazole** [153] and **Artemisinin,** and its derivatives, are other repositioned drugs currently used. This one was first described for the treatment of Malaria, and later expanded to some cancers, metazoan helminths, fungi and some viruses. More recently it was proposed as possible therapeutic for pathogenic protozoa such as *Trypanosoma* spp. and *Leishmania* spp. [179-184]. As other approach, **inhibitors of Histone deacetylase (HDAC) enzymes**, which are clinically approved for T cell lymphoma, are being pursued as potential drug targets for Malaria, Trypanosomiasis, Leishmaniasis [185], and other parasitic diseases [186-187]. Completing the list, **Sulphonamide compounds**, primarily target for carbonic anhydrase, have also been studied as potential antimalarial [188-190], anti-trypanasomal and antileishmanial agents [191-193].

It is worth mentioning the new techniques that have been employed for high throughput screening (HTS) of drugs. Peña and coworkers reported from a HTS of the GlaxoSmithKline (GSK) compound collection (1.8 million compounds) against the three most relevant kinetoplastids causative of human disease: *L. donovani, T. cruzi and T. brucei*. Confirmatory antiparasitic assays were performed and the potential for non-specific cytotoxicity determined. Hit compounds were chemically clustered and triaged for desirable physicochemical properties. These diversity sets were investigated through bioinformatics methodologies. Based on

conservative thresholds, $pIC_{50} \geq 5$ for inhibition or antagonist assays and $pEC_{50} \geq 5$ for agonist activation or modulator assays, compounds were associated with human protein targets which were used as BLASTP queries against the respective parasite genome. The number of compounds meeting target specificity criteria was similar across *L. donovani* (80 compounds) and *T. brucei* (82 compounds) but lower for *T. cruzi* (46 compounds). Functional analyses of these compounds suggest a wide array of potential modes of action against kinetoplastid kinases, proteases and cytochromes as well as potential host–pathogen targets. The compound sets are provided as an open resource for future lead discovery programs, and to address important research questions [194].

5. CONCLUSIONS

Closing this review, it is worth mentioning that the three tropical protozoan diseases addressed here have suffered a similar fate: while for many years, available therapies have been insufficient in number and effectiveness, in the last two decades there has been a boom of initiatives applying drug repositioning and other approaches, opening new therapeutic alternatives. Noteworthy, the vast majority of new treatments at clinical stage of development correspond to repurposed candidates. This cost- and time-efficient drug development approach has allowed reaching new options that are currently in use in the clinical practice, and finding a significant number of potential new therapies, some of them undergoing clinical trials.

Attention is still required in two points: one referred to divergence and variability of kinetoplastid parasites that could be determinant in therapy effectiveness, as it was shown in *T. cruzi* [72-73]; the second, referred to the actions to improve current predictive models of infection, perform pharmacokinetic studies and develop diagnostic tools that would expedite the evaluation of repurposed drug candidates and the interpretation of clinical trials results. At present, different consortiums, public-public or public-private, as Drugs for Neglected Diseases initiative (DNDi), provide platforms to enable progression of compounds into and through clinical trials [195]. Present efforts towards new medications and diagnostic tools taking place in endemic countries deserve to be highlighted; owing to limited resources, such efforts need to focus on cost-effective, ingenious solutions.

Suitable new therapies for the trypanosomatid diseases should gather a series of features, including selectivity, safety, oral bioavailability, accessible costs, sustainable production, and the possibility of taking treatment in circumstances of limited access to health facilities and medical surveillance. Appropriate selection of repurposed candidates can certainly fulfill at least some of these requirements. It can be expected that in a few more years there will be adequate therapies for different parasite strains and disease states, something unconceivable twenty years ago.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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