Topics in Medicinal Chemistry 39

Alane Beatriz Vermelho
Claudiu T. Supuran *Editors*

# Antiprotozoal Drug Development and Delivery

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# Antiprotozoal Drug Development and Delivery

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# Preface

Protozoans are microscopic, nonfilamentous protists belonging to a multitude of phyla, with several genera and species described so far, many of which possess ecological and industrial importance. However, they may also produce diseases in vertebrates, which may range from mild to moderate, such as those induced by *Toxoplasma gondii* or *Entamoeba histolytica*, to more serious conditions (infections due to *Cryptosporidium parvum*, *Giardia lamblia*, *Trichomonas vaginalis, Babesia* spp.) or to severe and widespread ones, such as malaria, leishmaniasis, Chagas disease, and African sleeping disease. Although rare, there are also several fatal protozoal diseases, such as those induced by amoebae belonging to the following genera/species: *Naegleria fowleri, Acanthamoeba* spp., and *Balamuthia mandrillaris.*

Few effective therapeutic approaches are available to treat most diseases provoked by protozoans. Although all 12 protozoans genera that elicit human disease are now well studied, there are very few drugs useful for treating these conditions. Furthermore, these drugs that have been available for many decades generally show high toxicity and low therapeutic indexes, and more concerning, there is an extensive resistance to these treatment options lately. Thus, in this book, we present a series of interdisciplinary reviews dealing with some of the most widespread protozoans that cause human disease, together with the latest drug design and pharmacological studies that have recently emerged to manage such diseases.

The first part of the book, comprising seven chapters, deals with some of the most widespread and difficult to treat pathologies caused by protozoans, specifically, infections due to *Leishmania* spp., *Trypanosoma cruzi* (Chagas disease) and *Trypanosoma brucei* (African sleeping disease). The first chapter by Nico and collaborators presents an exhaustive and updated review of the classical as well as new studies of drugs design for the management of *Leishmania* infections, whereas the second chapter by Amaral et al. presents in detail the saponins as a potential class of compounds possessing anti protozoan efficacy.

The third chapter by Vermelho and collaborators exhaustively presents Chagas disease, the currently used drugs (in fact, only two such compounds are available),

the new pharmacological targets identified so far, and the corresponding drug design approaches for the management of *T. cruzi* infection. Along the same line, the fourth chapter, by Nocentini et al. describes the carbonic anhydrases from *T. cruzi* and *Leishmania* spp. as new and potentially relevant anti-protozoan drug targets, considering the important advances which have been registered in the last decade for finding inhibitors for these enzymes and for validating them as drug targets. The fifth chapter by Carradori et al. describes the infection and drug design studies for managing *Trypanosoma brucei* infection, which causes African sleeping disease. In fact, one of the few remarkable successes in the management of this disease is the recent approval of a new drug, Fexinidazole, which, although belonging to the well-known class of the nitroazoles, seems to possess a much higher efficacy compared to classical drugs, among which Nifurtimox, Benznidazole, Metronidazole, or Tinidazole. The sixth chapter by Rodrigues et al. presents the polyamine and trypanothone biochemical pathways for targeting protozoan infections (again mainly *Trypanosoma cruzi* and *Leishmania* spp. infections) and the drug design studies that emerged in the last period in the field, whereas the seventh chapter by Rossi-Bergmann and collaborators present an updated review on the nano- a micro-systems for the drug delivery of anti-leishmanial drugs.

The next section of the book, comprising two chapters, deals with one of the worst protozoan infections, malaria, caused by five *Plasmodium* species that infect humans (*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*). As well known, the parasite is transmitted by mosquito bites, the infection is widespread in tropical countries, there are few effective therapeutic options, a high drug resistance problem to the currently available agents, and a large number of casualties due to this infection. The eighth chapter by Mori and collaborators exhaustively presents the intricate life cycle of the parasite, the various approaches to target it, the currently available drugs, and new drug design strategies reported ultimately, although no new antimalarial drugs emerged in the last three decades. In the ninth chapter, Capasso and Supuran present the η-carbonic anhydrases, a class of enzymes discovered in 2015, as potential new antimalarial targets, with all the numerous studies performed so far for identifying selective and effective inhibitors as well as the efforts to validate these enzymes as anti-protozoan drug targets.

The last section of the book, comprising four chapters, deals with the protozoans causing milder infections compared to the parasites dealt with in the previous chapters, more precisely *Entamoeba histolytica* (tenth chapter by Parkkila and Haapanen), *Trichominas vaginalis* (11th chapter by Parkkila, 12th chapter by De Simone et al.), and *Toxoplasma gondii* (13th chapter by Guglielmi and Secci). In all of them, the state-of-the-art regarding the treatment as well as the few drug design studies that emerged ultimately is presented in detail.

The last chapter of the book, by Vermelho et al., presents a detailed analysis of the field, stressing the fact that the development of anti-protozoal drugs has been hindered by several factors, among which the complicated lifecycles of such organisms and their ability to avoid innate immune defences; challenges associated with culturing protozoans, particularly in different phases of their growth and

amplification; and the lack of investment in biomedical research aimed at developing treatments for tropical diseases that do not tend to affect more affluent countries.

Overall, we estimate that the present book will stimulate the interest of students, researchers, and specialists in many interdisciplinary fields from Academia and pharmaceutical industries, which might lead to a better understanding of all challenges connected to the discovery and development of new anti-protozoan drugs, which on the other hand are urgently needed.

The editors wish to express their gratitude to the coworkers and colleagues who contributed to the book with high-quality manuscripts.

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# Classical and Modern Drug Treatments for Leishmaniasis


**Dirlei Nico, Luciana Conde, and Clarisa Beatriz Palatnik de Sousa**

## Contents



**Abstract** Leishmaniasis is a complex disease caused by intracellular parasites of the genus *Leishmania* spp. According to the World Health Organization (WHO) there are over one billion people at risk of infection. In more than 95% of the cases, visceral leishmaniasis is fatal if not treated. Antimonials are used as the first-choice

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drug to control leishmaniasis; however, their use is limited owing to high toxicity, parenteral administration, and monitoring throughout the entire treatment. The resistance to antimonials, most notably in India, has become a serious problem and has led to use of alternative drugs such as amphotericin B, pentamidine, and miltefosine, which are considered to be second-choice drugs. The lipid formulations of amphotericin B were successfully developed to decrease toxicity; miltefosine is an oral drug that succeeded in India; however, the efficacy of this drug in other countries still shows conflicting results. Drug combinations have been tested to minimize side effects and decrease duration of treatment and cases of drug resistance. There are a few optional therapies, but no vaccines against human leishmaniasis until now. In this review, we discuss current and new drugs and the priority of establishing new strategies for the treatment of leishmaniasis.

**Keywords** Amphotericin B, Antimonials, Drugs from natural sources, *Leishmania*, Leishmaniasis, Miltefosine

## 1 Introduction

*Leishmania* spp. are protozoan parasites of the Trypanosomatidae family (Kinetoplastida) characterized by the presence of a kinetoplast and a unique mitochondria [1]. Parasites of the genus *Leishmania* cause leishmaniasis, a complex disease with different symptomatic manifestations and can present as the cutaneous or visceral forms of the disease. More than 20 disease-causing species have been described. According to the World Health Organization (WHO), leishmaniasis is an infectious and tropical disease that occurs globally and is one of the top 10 neglected diseases [2]. Leishmaniasis has been reported in 102 countries and threatens over one billion people living in risk-prone areas, with both cutaneous and visceral leishmaniasis. Visceral leishmaniasis (VL) is the most severe form of the disease since the protozoan parasite affects various organs in the vertebrate host, mostly the liver and spleen, causing hepatosplenomegaly and is fatal without adequate treatment [3]. More than 90% of the global VL cases occur in six countries: India, Bangladesh, Sudan, South Sudan, Brazil, and Ethiopia; the overall annual incidence of VL is between 202,200 and 389,100 cases [4]. However, in the case of cutaneous leishmaniasis (CL), the most common form of the disease, the highest incidences are found in Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, North Sudan, Costa Rica, and Peru. Collectively, these countries account for 70–75% of the cases worldwide. The annual global incidence is reportedly between 690,900 and 1,213,300 cases per year [2, 4].

Leishmaniasis is caused by the internalization of parasites by phagocytosis via CR1 (first complement receptor), CR3 (third complement receptor), MR (mannose receptor), and FcγR (Fc gamma receptor) present in macrophages of the vertebrate host, as shown in models of *Leishmania major* infection [5, 6]. The parasite is

transmitted to the vertebrate host through the bite of the sandfly during the blood meal of females. The life cycle of *Leishmania* is composed of two different morphological stages: the amastigote and promastigote (either procyclic or metacyclic) forms. The metacyclic promastigote forms are characterized as a mobile, flagellated, and non-replicative form, found in the proboscis of the sandflies and are inoculated together with the saliva when the insect bites the vertebrate host. Characteristically, the metacyclic promastigote forms are smaller than the procyclic forms, which are the replicative form found in the gut of the insect [3, 7]. Amastigote forms replicate within the parasitophorous vacuoles (PVs) and do not exhibit motility [8]. During the blood meal, female sandflies inject the metacyclic forms with anticoagulant substances into the saliva of the vector, an important mechanism facilitating the entry of these forms into the vertebrate host [9, 10]. Within mononuclear phagocytic cells, the metacyclic forms undergo morphological changes into amastigote forms that remain within the PVs and multiply by binary division until they induce lysis of the host cells. The subsequent release of amastigotes infects new cells of the mononuclear phagocytic system [9, 11, 12]. The cycle continues when females of the insect vector consume a blood meal and ingest the amastigote forms from an infected vertebrate host.

Leishmaniasis can be grouped into three distinct clinical manifestations: CL, mucocutaneous leishmaniasis (MCL), and VL, also known as kala-azar [4, 13]. CL and MCL are also called tegumentary leishmaniasis (TL). CL, the most common form, promotes ulcers on the exposed parts of the body, that may heal after a few weeks [14]. However, the ulcers may lead to serious aesthetic problems due to formation of scars on the skin. MCL usually promotes partial or complete destruction of the mucous membranes of the oral and nasal cavities, affecting the mouth, nose, and throat [7, 14]. Clinical signs such as fever, weight loss, and anemia are observed in infected individuals [2]. In India and Africa, as a sequel of VL after treatment, post-kala-azar dermal leishmaniasis (PKDL) may manifest as innumerable nodules all over the body of the host. This clinical form is associated with *L. donovani* [14].

*Leishmania* parasites exhibit important mechanisms to evade the immune system in the vertebrate host, thus, guaranteeing their survival. When activated, macrophages produce proinflammatory cytokines that induce a Th1 immune response combating intracellular microorganisms [15]. The production of IFN-γ by CD4+ T cells is an essential defense against these microorganisms, in addition to the production of nitric oxide by macrophages [16, 17].

A dangerous scenario is one of co-infection with the human immunodeficiency virus (HIV) [18]. Co-infection with HIV increases the incidence of VL, and HIV infected individuals are more vulnerable to VL infection, with the progression from HIV to AIDS accelerated by increased viral replication in the host. Notably, the highest number of cases of HIV and HIV co-infection occur in Brazil, Ethiopia, and India. In Brazil, several cases of HIV co-infection with CL have also been reported [2, 19, 20].

Understanding the cellular and humoral immune response that develops during the treatment of leishmaniasis is of great importance in the study of the criteria that

can help improve the therapeutic results against the disease. Likewise, it could help explain the success or failure of some drugs associated or not with events of drug resistance. IFN-γ production is associated with a TH1 profile response which is associated to a successful outcome of VL treatment [21]. Pentavalent antimonials have shown less efficacy in the treatment of immunosuppressed patients with a HIV/VL co-infection. In addition, these patients have a greater number of adverse reactions due to toxicity and deaths [22]. Successful treatment of VL using amphotericin B is associated with an efficient TH1-type response [23]. The use of miltefosine is also associated with the activation of the TH1-type immune response, by increasing the production of IFN-γ, TNF-α, and IL-12 [24]. On the other hand, there are no reports that paromomycin activates the immune response and thus directly influences the treatment [25].

The treatment of leishmaniasis is a major challenge and should be initiated only after confirmation of a laboratory diagnosis [2]. There are a limited number of possible therapies, which are part of different national and regional protocols according to the geographic region of the disease. However, although different species are responsible for different clinical manifestations, the same drugs are available for the treatment of VL and CL [26]. In the case of CL, the choice of drugs, used in the treatment, is dependent on the geographic location of the individual, as well as the presence of any comorbidities; there is no standard global protocol [27]. Antimonials have been the first-choice treatment for approximately 70 years. These medications require the patient to be hospitalized for the drug administration and daily monitoring for any adverse reactions. The debilitated conditions of many patients with VL such as malnutrition and anemia further complicate treatment. These conditions could induce extremely serious adverse effects and even death. In some cases, clinical support such as hydration and nutritional supplementation is necessary before initiating treatment. This is an important strategy since leishmaniasis is associated with countries with low socioeconomic development and poor sanitary conditions. This practice minimizes the incidence of death during and after disease treatment [28].

The difficulty in establishing a single protocol for the treatment of leishmaniasis is related to the difficulty in eliminating the parasite. This is due to its complex life cycle and its high capacity to evade the immune system. The drugs currently used, which we will call current or classic drugs, are divided into first- and second-line choices. These drugs vary between the protocols published by competent organs in different countries. This chapter will address the mechanisms of action of the classic drugs that are currently in use and the new drugs that are emerging as an option in the fight against this disease. Furthermore, we will discuss the need to establish new strategies for the treatment of leishmaniasis.

## 2 Current Drugs in Use for the Treatment of Leishmaniasis

The treatments in use against leishmaniasis can be classified into drugs of first-choice, drugs of second-choice, and other drugs (Table 1). They are licensed drugs and with therapeutic protocols recommended by WHO (Table 2). Unfortunately, the scenario that is presented below is extremely restricted, with serious and unpleasant adverse effects limiting the patient's adherence to treatment. The first of these classical drugs was introduced over 100 years ago and even the first-choice drug of today was introduced over 50 years ago (Fig. 1). Therefore, the principal concepts must be revisited and reflected on to find new therapeutic alternatives that are more effective and without such adverse effects.

**Table 1** Classical drugs used in the treatment of VL in most countries

| First-line | Second-line | Others drugs approved |
|---|---|---|
| Pentavalent antimonials | Amphotericin B deoxycholate | Lipid formulations of amphotericin B |
| | Pentamidine | Miltefosine |
| | | Paromomycin |

**Table 2** Dosages of first-line and second-line treatments for VL [42]

| Drug | Dosage | Period |
|---|---|---|
| Pentavalent antimonials | 20 mg/kg | Single dose |
| | (Upper limit of 850 mg) | |
| Miltefosine | 2.5 mg/kg to children | 28 days |
| | 50 mg/day <25 kg | |
| | 100 mg/day >25–50 kg | |
| | 150 mg/day >50 kg | |
| Amphotericin B deoxycholate | 0.75–1 mg/day | 15–20 days |
| Liposomal amphotericin B | India: >10 mg/kg | Single dose |
| | Southern Europe: 3–4 mg/kg to 15–24 mg/kg | |
| | Mediterranean: 20 mg/kg | 2 doses |
| Paromomycin | India: 15 mg/kg | 21 days |
| | East Africa: 20 mg/kg | |

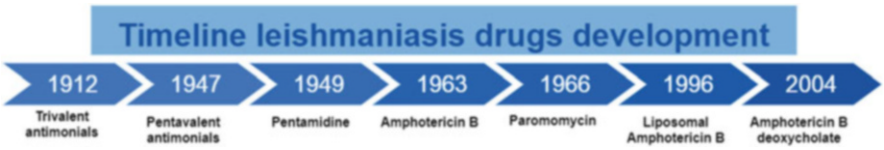


**Fig. 1** Leishmaniasis drug development timeline. The year of introduction of the drugs used to treat leishmaniasis across the world

## 2.1 Antimonials

Initially, trivalent antimonials ($Sb^{3+}$) were used against leishmaniasis by Gaspar Vianna in 1912 [23]. However, owing to severe adverse effects, trivalent antimonials were discontinued and replaced with pentavalent antimonials ($Sb^{5+}$) in the late 1940s, and these remain the first-choice drugs until today (Table 2) [29, 30]. Pentavalent antimonials, since their introduction in the treatment of leishmaniasis, have shown excellent results and successful cure rates of around 90% have been observed [31]. Even in East Africa, therapies with antimonials have demonstrated cure rates of 93.9% in patients with VL [32]. However, drug resistance has become a major concern in India and Nepal, where more than 60% of unresponsiveness has been reported [33].

Pentavalent antimonials are used in clinical practice with the following formulations: meglumine antimoniate and sodium stibogluconate [34]. However, these drugs cause serious adverse reactions such as nephrotoxicity [35], hepatotoxicity [36], and cardiotoxicity [37] that need daily monitoring. Despite this, pentavalent antimonials have assumed the position of first-line drugs in systemic treatment in most countries [38]; however, CL treatment depends on specific protocols in different countries. Although used globally in the treatment of leishmaniasis, the mechanism of action of pentavalent antimonials has yet to be fully elucidated; several underlying mechanisms have already been proposed.

Pentavalent antimonials have been described as behaving as prodrugs. $Sb^{5+}$ enters in the amastigote form and is reduced to $Sb^{3+}$ [39–41], which is an oxidative state markedly effective in combating the intracellular parasites of *Leishmania.* This reduction has only been observed in the amastigote forms, and therefore, it is stage-specific [40–42]. In fact, the pentavalent antimonial (Glucantime®) treatment promoted a decrease of parasite burden when compared to the untreated control groups [43]. After reduction to the $Sb^{3+}$ form, parasitic trypanothione and trypanothione reductase are inhibited, which is an important molecular mechanism of action of antimonials [44, 45]. Baiocco et al. showed the structural analysis of trypanothione reductase with NADPH and $Sb^{3+}$, which determined the molecular basis in which $Sb^{3+}$ inhibits the action of trypanothione reductase [43, 44, 46].

In spite of this elimination of *Leishmania* forms with pentavalent antimonials, cases of resistance to pentavalent antimonials were reported in India in the 1980s [47]. At this point, changes were already introduced in the treatment protocols for Indian Kala-azar, resulting in concerns regarding the new treatment for leishmaniasis; this problem is even greater today. In CL, the treatment consists of a local injection of pentavalent antimonials in milder cases and systemic treatment in severe cases [48]. In parallel to treatment with antimonials, the liver function, blood cell counts, serum amylase, and lipase levels should all be monitored.

Local treatment of lesions (intralesionally) caused by cutaneous leishmaniasis is an attractive option for the patient, since there is no need for hospitalization and the toxic effects of systemic origin caused by intravenous treatment are reduced [49]. A disadvantage is local pain of greater intensity at the time of administration of the

drug [50]. Pentavalent antimonials, when administered intralesionally, require a varying number of sessions and different quantities per application, depending on the patient's clinical response. Most patients generally require less than five sessions of pentavalent antimonials containing 1–5 ml per session [49]. This treatment has shown different cure rates around the world. In Iran against *L. major*, this type of local treatment had a cure rate of 56% [51], in Brazil against *L. braziliensis* the cure rate was 80% [52].

## 2.2 *Amphotericin B Deoxycholate and Liposomal Amphotericin B*

Amphotericin B deoxycholate and pentamidine are characterized as second-line drugs in the treatment of leishmaniasis [33]. Amphotericin B deoxycholate is a polyene medicine, with an extremely affordable formulation that promotes a high cure rate; however, the main contraindication associated with its use is nephrotoxicity [53], consequently resulting in treatment discontinuation. Furthermore, amphotericin B treatment is administered via the parenteral route, requiring patient hospitalization. In addition, changes in glomerular filtration and damage to the tubular plasma membranes have been observed during treatment, causing problems in the reabsorption of electrolytes [54, 55].

Alternative formulations of amphotericin B such as liposomal amphotericin B, amphotericin B lipid complex, and amphotericin B colloidal dispersion have been proposed. These formulations demonstrate lesser toxicity than and efficacy similar to amphotericin B deoxycholate [33]; thus, presenting less nephrotoxicity [56]. Several protocols with different concentrations have been proposed, presenting cure rates above 90% in cases of VL [57]. Efficacy rates in the treatment of VL vary according to geographic location. A high dose of liposomal amphotericin B has been reported to achieve more than 90% success in the treatment of VL. Using AmBisome (liposomal amphotericin B) 10 mg/kg has reached 95% cure rates in India and 30 mg/kg has shown 92% cure rates in Sudan. On the other hand, amphotericin B deoxycholate (AmB) in India has shown 95–100% effective after 15–20 infusions of the drug with a dose of 0.75–1.0 mg/kg [58, 59].

The underlying mechanism of amphotericin B action is associated with its high affinity to the *Leishmania* cell membrane ergosterol [60]. Thus, canals are formed due to the complexation between amphotericin B and cholesterol [61]. Liposomal amphotericin B decreases the nephrotoxicity of amphotericin B, which is one of the major causes of treatment discontinuation [53, 62]. The lower toxicity has been attributed to a reduced interaction with human cell membranes. Amphotericin released from the liposome would bind to ergosterol in leishmaniasis. Thus, aqueous pores are formed by increasing cell permeability. Another proposed mechanism of action is the interaction of *Leishmania* ergosterol, macrophage cholesterol, and amphotericin B, which would negate the macrophage parasite interaction

[63]. Table 2 also lists the therapeutic schemes of amphotericin B and liposomal amphotericin B for VL.

## 2.3 Pentamidine

Pentamidine is an aromatic diamine that is administered parenterally and has limited use owing to the serious adverse effects induced in patients; especially those with diabetes mellitus, hypoglycemia, nephrotoxicity, and myocarditis [33]. In India, this drug can be used as an alternative treatment against VL [64] since there has been resistance to pentavalent antimonials and pentamidine presents a treatment option with superior results [65]. Additionally, it was observed that the use of a treatment combination of pentamidine and miltefosine reached an impressive cure rate of 92% in patients infected with *L. braziliensis* in Bolivia [66]. In Brazil, the use of a single dose of pentamidine (7 mg/kg) against CL caused by *L. guyanensis* has shown promising results [67]. Pentamidine was also evaluated in VL treatment and shows a 70–80% efficacy rate using a dose of 4 mg/kg/day three times a week, totaling 15–20 doses [58].

Although the mechanism of pentamidine has not been fully elucidated, its mechanism has been shown to rely on the leishmanicidal activity induced by *Leishmania* apoptosis [68]. This drug can interfere in parasite DNA synthesis, promoting changes in kinetoplast morphology and the mitochondrial membrane [26]. As shown in antimonials, pentamidine demonstrates a T cell-dependent immune response [69].

## 2.4 Paromomycin

Paromomycin is a broad-spectrum aminoglycoside antibiotic originating from the bacterial pathogen *Streptomyces rimosus* var. *paromomycinus*. It is active against Gram-negative and Gram-positive bacteria, some protozoa, and cestodes [70]. Although it was first discovered in the 1950s, it was only introduced for VL treatment in 2006 [71]. Paromomycin can be administered as 250 mg capsules for oral intake, or intramuscularly. However, 2% of patients treated with paromomycin demonstrate reversible ototoxicity and, in some cases, hepatotoxicity [72, 73]. Some variation in efficacy can be observed using paromomycin against VL using the dose of 11 mg/kg/day for 21 days, which has shown in India more than 95% efficiency while in Africa it has shown a variation of 46–85% efficiency [58]. Furthermore, an inhalational form of paromomycin has been indicated for systemic infections [74]. A variant of the topical formulation can be used in the treatment of TL [33]. The paromomycin mechanism of action involves mitochondrial alterations that modify the energetic metabolism resulting in apoptosis [75]. A topical use of paromomycin has been suggested since the 1980s for the treatment of cutaneous leishmaniasis;

however, its power to penetrate the lesion depends on the type of formulation and the use of substances that improve the absorption of the drug at the lesion site [76]. The use of paromomycin in different formulations in combination with antimonials via the intravenous route also showed different efficacies in Colombia [77].

In a first study using paromomycin as the treatment for VL, Buffet et al. demonstrated its good efficacy in an *L. infantum* experimental infection [78]. Subsequently, additional information was published confirming the antileishmanial activity in vivo and in vitro [79]. Clinical studies reported therapeutic efficacy in humans [80–82] and parasite clearance occurred within 6–7 months after drug administration [72].

## *2.5 Miltefosine*

Miltefosine (hexadecylphosphocholine) is an important oral drug in the treatment of leishmaniasis. Initially developed as an anticancer drug [83], miltefosine induces several adverse effects; mainly gastrointestinal reactions and teratogenic actions. Miltefosine is the first oral drug that induces leishmanicidal activity [84]. Vincent et al. performed a metabolomic study on *L. infantum* (strain JPCM5) promastigotes, evaluating the multiple mechanisms of miltefosine actions and its leishmanicidal power by observing the metabolic alterations. Furthermore, extensive DNA damage caused by the leishmanicidal mechanism was detected. After 5 h treatments with miltefosine, changes were observed in cell membranes, with metabolites escaping after cell death. In promastigotes, the suggested mechanism of action of miltefosine is cell lysis, preceded by changes in lipid metabolism that promote an increase in alkanes, sugars, and nucleotides, owing to the induction of toxic reactive oxygen species (ROS) [85]. Miltefosine acts on the lipid content of the cell membranes of promastigote forms by altering the content of membrane phospholipids, phosphatidylethanolamine, and lysophosphatidylcholine [86]. Miltefosine also acts on macrophages [85, 87]. Additionally, modifications induced in the composition of phospholipids, fatty acids, and sterols have been demonstrated in the promastigote membranes of *L. donovani* [86]. Miltefosine, antimonials, and amphotericin B cause apoptosis associated with ROS [68]. Table 2 shows the therapeutic scheme of miltefosine for VL.

CL can be treated with different types of therapeutic approaches. According to WHO, the classical treatment against CL encompasses topical and systemic options. Based on the literature, it is evident that a fixed protocol is lacking and treatments differ according to the geographical locations, protozoan species involved, the response of the infected host, and host comorbidities [88]. As CL does not cause serious complications beyond esthetic damage, the treatment decision depends on the economic capability of each patient, and availability in his location/country. For example, if the patient has numerous lesions with severe disfiguration to the face or other areas on the body difficult to access using local therapy, systemic treatment should be considered. Similarly, if the infected patient has serious comorbidities (cardiac, hepatic, renal, pancreatic, or hematological), a safer treatment with no

**Table 3** Treatment of leishmaniasis during pregnancy [42]

| Amphotericin B deoxycholate and lipid formulations | Pentavalent antimonials | Paromomycin | Pentamidine | Miltefosine |
|---|---|---|---|---|
| The best therapeutic options for VL | Less safe | Ototoxicity in the fetus | Contraindicated during the first trimester | Embryotoxic and teratogenic should not be used |

serious adverse reactions should be considered [33]. The efficacy in the VL treatment with miltefosine presented an efficacy rate varying from 85 to 95% using the dose of 150 mg/day in adults weighing more than 50 kg [58].

During pregnancy, the treatment of leishmaniasis has markedly severe limitations, especially in the case of VL that requires systemic treatment. However, CL treatment can be achieved with local therapies. Table 3 shows the best treatment option during pregnancy and its main adverse effects.

## 3 New Drugs Available for Leishmaniasis Treatment

There are only a few new drugs available and approved treatments for clinical use, which are costly and induce several adverse effects with severe intensities. However, with scientific and technological advances in the field of bioinformatics, parasite genomics, and microscopy, the research and development of new drugs has gained considerable momentum. One excellent way for the development of new drugs is to identifying potential molecular targets in the parasite [89]; however, this is beyond the scope of this survey.

### *3.1 Drug Delivery Systems*

Drug delivery systems (DDS) are excellent tools for the development of new drugs. There are different types of DDS, including liposomes, niosomes, and nanoparticles [90]. Nanotherapy addresses the use of nano DDS to improve the performance of drugs utilized in clinical therapy against various diseases. This important tool has potential in the treatment of leishmaniasis. Using this approach, the drug can be directed toward internalization in the target cell or to the target organ [91–93]. An example of the efficient use of this tool was provided in the study by Kalangi et al. [93], in which silver nanoparticles (AgNPs) were used to improve the therapeutic efficiency of miltefosine and can possibly be applied to reduce the drug concentration.

### 3.2 Immucillins

Immucillins are synthetic drugs that have shown great versatility in experimental and clinical trials in the treatment of several diseases caused by various etiological agents [94]. Among them are the activity against *Plasmodium falciparum* [84]; *L. infantum chagasi* [95]; *L. amazonensis* [43]; *Helicobacter pylori* [96]; additionally, potential antiviral effects were observed against serious viruses, including Ebola, yellow fever and Zika virus [97].

In terms of the therapeutic potential against *Leishmania* species, immucillin has proven to be an excellent alternative for therapeutic use [43, 95]. Protozoa of the *Leishmania* genus are known to obtain purine bases from exogenous precursor sources and use the purine salvation pathway to accomplish this task [98, 99]. Nucleoside hydrolases (NH) are enzymes capable of hydrolyzing nucleosides and thus releasing purine bases for use in parasite DNA synthesis, as demonstrated in *Leishmania*. NH are absent in human cells and this is the important factor to be considered for therapeutic use [100]. Immucillins IA and IH, according to studies by Freitas et al. [43], were the two immucillins that presented the highest NH inhibition profiles in vitro. Based on this result, immucillins inhibited the growth of *Leishmania* parasites in vitro.

### 3.3 Drugs from Natural Sources

Medicinal plants have important active substances that can be used in the treatment of leishmaniasis [101, 102]. Plant extracts contain various chemical groups with distinct medicinal properties. Among them, alkaloids demonstrate therapeutic potential against VL [103]. Furthermore, in the case of CL, several other plants are potentially promising and have been proven efficacious [101].The plants of the *Asteraceae* family, genus *Artemisia*, showed important leishmanicidal effects [104, 105]. Additionally, garlic extract has shown a leishmanicidal effect [106].

*Kalanchoe pinnata* is a medicinal plant with potential leishmanicidal activity against different types of leishmaniasis in the murine and humans models [107–109]. In CL induced by *L. amazonensis*, oral treatment with *Kalanchoe pinnata* is as effective as the classical treatment with pentavalent antimonials [107]. However, in the treatment of a properly diagnosed human patient with CL, oral treatment with the *Kalanchoe pinnata* aqueous extract controlled the lesion during treatment; once treatment was discontinued, the lesion grew back. Oral treatment is safe and does not induce alterations in the renal and hepatic system; it also does not cause unpleasant adverse effects [109]. Therefore, it is an important medicinal plant with therapeutic potential against VL and CL, with the advantage of oral administration. There are many other medicinal plants with leishmanicidal properties that can be found in area-specific reviews.

## 4 Combination Therapy

In the case of VL, some combination treatment schemes have been proposed and tested. Combination therapies are necessary to prevent the emergence of resistance to the drugs in use. One such recommended combination is paromomycin–miltefosine, which is yet to undergo a systematic quality assurance investigation. Another combination undergoing trials in India and Bangladesh is amphotericin B-miltefosine, which has a shorter duration of treatment; however, it demonstrates toxicity in patients with PKDL and HIV co-infection [110]. Furthermore, amphotericin B requires quality control assurances for its storage. In CL treatment, the combination of intralesional applications of $Sb^{5+}$ and cryotherapy was found to be an important alternative [111].

HIV-positive patients are a group of patients to whom a combination of therapies is of paramount importance as classical treatments are less effective and markedly toxic. An example of a successful drug combination for this group would be sodium stibogluconate and paromomycin [89]. Consistent improvements in post-treatment results have been achieved after using drug combinations, which makes them an excellent option for leishmaniasis treatments.

## 5 Alternative Therapies

### *5.1 Sitamaquine*

Sitamaquine is an alternative oral drug against VL [112, 113]. Phase II clinical trials in India and Africa have reported interesting results with short treatment durations, good results, and low rates of adverse effects [64]. Another Phase IIb trial demonstrated few indices of adverse effects and with no serious or irreversible hepatic, renal, or cardiac effects reported [113]. Furthermore, in vitro studies have demonstrated the leishmanicidal activity of this drug against CL [114]. In vitro studies using promastigote forms of *L. donovani* showed that the mechanism of action of sitamaquine involves morphological changes in the parasites, as well as changes in motility and growth, with an approximate 60% reduction in the parasitic load [112]. Human studies have shown that sitamaquine has an elimination half-life of 26 h, which prevents the appearance of drug resistance [115].

Therapeutic combinations with sitamaquine need to be investigated. Especially since sitamaquine combinations have been widely considered in theoretical solutions.

### 5.2 *Ketoconazole, Fluconazole, and Itraconazole*

Ketoconazole, fluconazole and itraconazole are antifungal agents that can be administered orally, exhibiting variable efficacy in the treatment of leishmaniasis [33]. The possibility of oral administration of these drugs is an excellent advantage and is attractive for patient treatment compliance. As antileishmanial drugs, they act similarly to amphotericin B, where they interfere with ergosterol synthesis [30]. The efficacy of these antifungal agents was high in the treatment of CL and low in the treatment of VL [116]. In India, oral itraconazole is effective against CL [117, 118]. Additionally, treatment with oral itraconazole is less toxic than ketoconazole [119]. One possibility for effective treatment against leishmaniasis would be the association between these antifungal agents and drugs of the first- or second-choice [120]. This association is primarily necessary when the therapy being used is ineffective.

### 5.3 *Physical Treatment*

In TL treatment, interesting alternatives, including physical treatment involving cryotherapy and thermotherapy, have been indicated. These alternatives have been recommended by WHO. This recommendation involves the application of liquid nitrogen for 20 s with a radiofrequency ablation device at 50°C for 30 s. Although thermotherapy is a simple physical method, the need for local anesthesia is considered a drawback as it makes the technique expensive [121, 122].

The objective of CL treatments is to accelerate healing and prevent lesion exacerbation. A successful alternative is the use of daylight-activated photodynamic therapy (DA-PDT) for lesions caused by *L. major* and *L. tropica* infection in adults and children; this treatment has better acceptance and tolerability in children than in adults. DA-PDT can also be used in combination with classical therapy as a treatment adjuvant, thereby reducing the treatment duration and dosage of the systemic medications [123].

## 6 Drug Resistance

The occurrence of resistance to treatment and, consequently, the selection of drug-resistant strains is a serious public health problem worldwide. In relation to drugs used against leishmaniasis, this problem is significant due to the limited number of drugs licensed for use in the clinic. Drug resistance generally involves genetic mutations that lead the microorganism to escape the mechanism of the drug used and therefore has no affect against the disease. As pentavalent antimonials are first-line drugs of choice, they are the main subject of this discussion point. Treatment

effectiveness needs to be monitored to detect any events of resistance as early as possible to avoid the appearance of new cases of drug resistance [124]. In addition, specific factors inherent to the host such as immunosuppression caused by HIV infection [125] and molecular mechanisms [126] are directly associated with the phenomenon of resistance to antileishmania drugs. In India, cases of visceral leishmaniasis present worrying levels of resistance to pentavalent antimonials. Interestingly, this may be related to the contaminated water that the population ingests with the heavy metal arsenic, leading to the selection of resistant parasites [127]. Cases of failure in treatment with antimonials in India reached 60%. This high level of failure promoted the withdrawal of pentavalent antimonials as the treatment of first choice and was replaced by amphotericin B [128, 129]. Therefore, different cellular and molecular mechanisms may be involved and mediate the emergence of drug resistance in eukaryotes such as *Leishmania.* The study of drug resistance is complex, and resistance to a single drug may involve different mechanisms; however, a more thorough and comprehensive approach to this topic regarding all drugs involved in the treatment of leishmaniasis is beyond our goal in this chapter.

## 7 Perspectives

Unfortunately, we have failed to develop a human vaccine for prophylactic use against leishmaniasis, although advances have been achieved toward a canine vaccine. Thus, the basic control is to prevent the sandfly insect vector bite and treat the disease with chemotherapy. Leishmaniasis treatment varies across continents and countries and presents several serious side effects that make it challenging to continue treatment, deeply impacting affected patients. Pentavalent antimonials are the drugs most used for the treatment of leishmaniasis treatment, and they have been the first-line of treatment for over 70 years.

The increasing number of drug resistance cases is a serious problem that needs to be urgently controlled. Furthermore, the limited number of possibilities for approved therapeutics is also concerning. Therefore, the introduction of new treatment possibilities is a fundamental issue that needs to be consolidated, widely discussed, and encouraged by development agencies. New efforts must be directed toward the development of novel drugs that do not have side effects, have a low cost, and are accessible to all, since cases of leishmaniasis occur worldwide. Considerable effort has also been made to redirect drugs from other diseases that present a leishmanicidal activity and some of these may present interesting alternatives.

An ideal drug should induce no adverse reactions or possess any risk to the patient, be of low cost, and have a short treatment duration. Furthermore, it is essential that the drug be administered through an easily accessible route. The oral route of administration is extremely attractive and has better adherence, as demonstrated in patients undergoing treatment.

## 8 Conclusion

The study and development of therapies – alone or in combination – that are safe and effective is an ongoing search for the treatment of leishmaniasis. The disease is a serious public health risk and its epidemiological profile has changed with the spread of the disease to areas previously free from infection. The scientific community should focus efforts on finding effective means to treat leishmaniasis and to evaluate new therapeutic strategies. Novel drugs that do not involve adverse effects and have low production costs are urgently required. Currently, several drugs are undergoing clinical trials and may provide potential future alternatives for the treatment of leishmaniasis.

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# Saponins as Potential Antiprotozoal Agents


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## Contents



**Abstract** The sphere of natural products is an abundant source for discovery of therapeutic drugs for the treatment of neglected parasitic diseases. Various classes of chemical substances displayed antiprotozoal activity, such as alkaloids, terpenoids, saponins, and flavonoids. The highly functional saponins are found predominantly in plants and are frequently consumed in foods, beverages, and medicines. This class of chemical substance has structurally one or more glycoside moieties linked to a triterpenoid or steroid. Saponins demonstrated to be very valuable therapeutic targets

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whose potential is still to be explored and that may be useful for the development of new antiprotozoal drugs options.

**Keywords** Natural products, Neglected disease, Protozoa, Steroid, Triterpenoid

## 1 Introduction

One of the main sources for the discovery of therapeutic targets for the treatment of neglected parasitic diseases is from natural products. Compounds isolated from plants, such as alkaloids, terpenoids, saponins, and flavonoids can display antiprotozoal activity. Saponins are highly functional and appear in more than 500 plant species, with up to 10% of saponin content per plant extract. These plant constituents are included in a large group of organic substances, which have in their molecular structures a steroidal or triterpenic nucleus (aglycone), most often linked to one or two glycoside radicals, each of which may contain one or more interlinked oses. The interaction mechanism of antiprotozoal saponins has an effect on the permeability of parasite cell membranes, causing vacuolization and disintegration of the teguments. Saponins appear to have a great potential that is yet to be explored and used in future research.

## 2 Saponin Structures

Saponins consist of two parts: an aglycone (also designated genin or sapogenin) and a glycone. The aglycone part characterizes the saponins that can be classified as triterpenoidal or steroidal. Triterpene saponins may contain oleanane, hopane, dammarane, or ursane skeletons, whereas steroidal saponins have spirostane, glycoalkaloid, or furostane in their skeletons (Fig. 1). Triterpenoid saponins are widely distributed in higher plants and the oleanane skeleton is the most common. Concerning glycone, L-arabinose, D-xylose, D-glucose, D-glucuronic acid, D-galactose, L-rhamnose, and D-fructose are sugars commonly found in saponins and may be associated with the aglycones through ester or ether bonds. Some additional classifications may be assigned to the saponins according to the position of the carbohydrate linkage in the aglycone chain, i.e., if the linkage occurs at the C3 position, the saponin is characterized as monodesmosidic whereas the saponins with an additional sugar moiety at the C26 position or C28 are called bidesmosidic. In some cases, a third sugar moiety in a different position of the aglycone forms a tridesmosidic saponin [1].

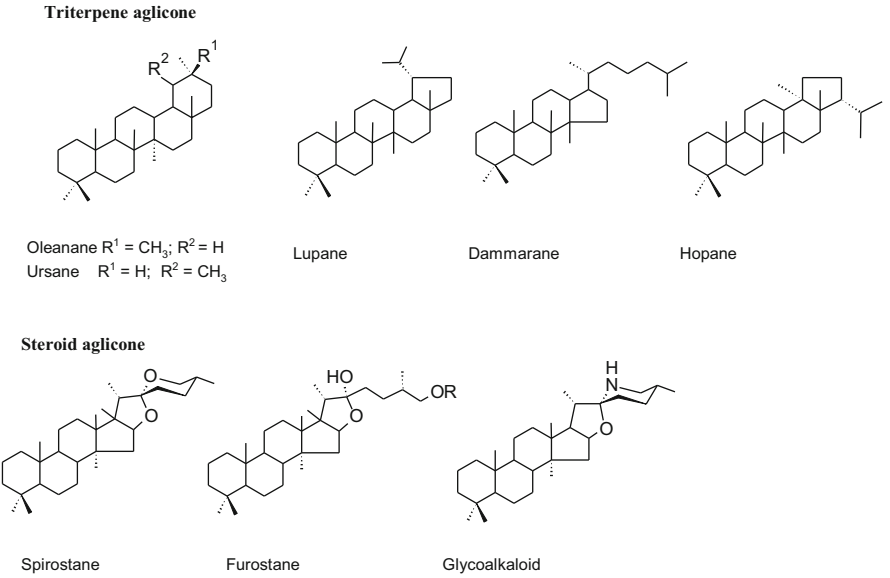



**Fig. 1** Basic skeletons of the triterpenoid and steroidal saponins aglycones

## 3 Distribution of Saponins in the Vegetable Kingdom

Saponins, which are natural organic substances, are found in a large group of constituents predominantly of vegetable origin but they can also be found in marine animals and have a characteristic of having afrosimetric properties, that is, producing abundant and persistent foam when shaken in water. Saponins are steroids or triterpenoids attached to one or more osydic units and/or derivatives. Thus, they are chemically divided into steroids, when the aglycone is a steroid (almost exclusively in monocotyledonous angiosperms, such as in the Agavaceae, Dioscoreaceae, and Liliaceae families), and triterpenoids when the aglycone is clearly a triterpene (mainly in the dicotyledonous angiosperms, such as in the Fabaceae, Araliaceae, and Caryophyllaceae families). Some plant species with saponins listed in Table 1 are used by man in his diet, as well as for medicinal purposes and in cattle feeding [2–4].

The pharmacological properties of saponins show that they have great therapeutic potential, including relevant antiprotozoal activity. In general, the saponins can form irreversible complexes with the cholesterol present in the cell membranes of protozoa leading to cell rupture and lysis. These effects, of course, will depend on the concentration and structural characteristics of the saponin, which due to its amphipathic nature can carry out the above-mentioned interactions, including the possibility of rearranging the lipid bilayer structure containing cholesterol leading to increased permeability of the cell membrane [5]. Table 2 presents the plants with saponins tested against different parasites.

**Table 1** Plant species with saponins traditionally used by man in food and as medicine

| Family | Botanic species (common name) |
|---|---|
| Amaranthaceae | *Beta vulgaris* L. (silver beet) |
| | *Chenopodium quinoa* Willd. (quinua) |
| | *Spinacia oleracea* L. (spinach) |
| | *Allium cepa* L. (onion) |
| | *Allium porrum* L. (leek) |
| | *Allium sativum* L. (garlic) |
| Araliaceae | *Panax ginseng* C.A. Mey. (ginseng) |
| Asparagaceae | *Asparagus officinalis* L. (asparagus) |
| Asteraceae | *Helianthus annuus* L. (sunflower) |
| Fabaceae | *Arachis hypogaea* L. (peanut) |
| | *Cicer arietinum* L. (chick-pea) |
| | *Glycine max* (L.) Merr. (soybean) |
| | *Glycyrrhiza glabra* L. (licorice) |
| | *Lens culinaris* L. Medik. (lentil) |
| | *Phaseolus mungo* L. (mung bean) |
| | *Phaseolus vulgaris* L. (bean) |
| | *Pisum sativum* L. (green pea) |
| | *Vicia faba* L. (broad bean) |
| | *Vigna angularis* (Willd.) Ohwi & H. Ohashi (azuki bean) |
| Lamiaceae | *Salvia officinalis* L. (sage) |
| | *Thymus vulgaris* L. (thyme) |
| Malvaceae | *Tilia europaea* L. (linden) |
| Myristicaceae | *Myristica fragrans* Houtt. (nutmeg) |
| Pedaliaceae | *Sesamum indicum* L. (sesame seed) |
| Poaceae | *Avena sativa* L. (oats) |
| Quillajaceae | *Quillaja saponaria* L. (quillaia bark) |
| Rosaceae | *Rubus* ssp. Hyb. (blackberry) |
| Smilacaceae | *Smilax aristolochiifolia* Mill. (sarsaparilla) |
| Solanaceae | *Solanum melongena* L. (eggplant) |
| Theaceae | *Thea sinensis* L. (tea) |

# 4 Pharmacological Activities of Saponins

## *4.1 Biological Activities*

Over the years, studies have shown various pharmacological activities related to saponins. A cytotoxic substance from *Allium chinense* saponins (ACSs), for example, has been used to test its anticancer activity against the B16 melanoma and 4T1breast carcinoma cell lines. The results showed that ACSs induced cell deaths in B16 melanoma and the 4T1 cells. In addition, the ACSs showed an inhibition of the melanoma growth in vivo [34].

**Table 2** Saponins with activity against parasites related to neglected diseases

| Botanical origin | Plant part | Parasite | Reference |
|---|---|---|---|
| *Asparagus africanus* Lam. (Asparagaceae) | Roots | *Leishmania major* | [6] |
| *Asparagus racemosus* Willd. (Asparagaceae) | Fruits | *Leishmania donovani* | [7–9] |
| *Brunfelsia grandiflora* D. Don (Solanaceae) | Leaves | *Leishmania major*; *L. guyanensis*; *L. panamensis* | [10] |
| *Calotropis procera* (Aiton) Dryand. (Asclepiadaceae) | Leaves | *Trypanosoma evansi* | [11, 12] |
| *Combretum leprosum* Mart. (Combretaceae) | Fruits | *Leishmania amazonensis* | [13] |
| *Eclipta prostrata* (L.) L. (Asteraceae) | Leaves | *Leishmania major*; *L. aethiopica*; *L. tropica* | [14–16] |
| *Glinus oppositifolius* (L.) Aug. DC. (Molluginaceae) | Aerial parts | *Leishmania donovani*; *Plasmodium falciparum* | [3, 17, 18] |
| *Hedera colchica* (K. Koch) K. Koch (Araliaceae) | Leaves | *Leishmania infantum*; *L. mexicana* | [7, 19–21] |
| *Hedera helix* L. (Araliaceae) | Leaves | *Leishmania infantum*; *L. tropica*; *L. mexicana*; *Trypanosoma brucei brucei*; | [3, 7, 9, 19–22] |
| *Ilex laurina* Kunth (Aquifoliaceae) | Leaves | *Leishmania panamensis* | [23] |
| *Maesa argentea* (Wallich) ADC. (Myrsinaceae) | Leaves | *Leishmania infantum*; *Plasmodium falciparum* | [7, 9, 24] |
| *Maesa balansae* Mez (Primulaceae) | Leaves | *Leishmania donovani*, *L. infantum* | [7, 21, 25–28] |
| *Maesa lanceolata* G. Don (Primulaceae) | Leaves | *Leishmania donovani* | [25, 27] |
| *Maesa sinensis* A. DC. (Primulaceae) | Leaves | *Leishmania donovani* | [25] |
| *Mussaenda luteola* Delile (Rubiaceae) | Aerial parts | *Leishmania donovani*; *Trypanosoma brucei brucei* | [29] |
| *Pfaffia glomerata* (Spreng.) Pedersen (Amaranthaceae) | Roots | *Trypanosoma cruzi* | [30] |
| *Plumbago capensis* Willd. (Plumbaginaceae) | Roots | *Leishmania major* | [31] |
| *Sapindus rarak* DC. (Sapindaceae) | Seeds | *Trypanosoma cruzi* | [32] |
| *Yucca schidigera* Ortgies (Asparagaceae) | Whole plant | *Giardia lamblia*; *Coccidia*, *Sarcocystis neurona* | [33] |

Another study, along these same research lines, suggested that a saponin extract from *Panax notoginseng* has potential to prevent cancer, including breast cancer. In order to evaluate the in vivo antitumor potential, the extract was administered orally to rats induced with mammary carcinogenesis for 30 days. In vitro tests have been made against human lung cancer NCI-H460 and breast cancer cell lines BT474 and analyzed using MTS assays [35].

Other triterpenoid saponins have also demonstrated cytotoxic activity against some human cancer cell lines. Two of them, known as (20S*,24R*)-epoxy-9β,19-cyclolanostane-3β,16β,25,28-tetrol-3-*O*-β-D-glucopyranoside and 3-*O*-[(6-*O*-n-butyl)-β-D-glucuronopyranosyl]-12-en-olean-3β,16β,28-triol were obtained from the seeds of *Ligularia przewalskii* and tested against HeLa, HepG2, SGC7901, MDA231, HL60, and Lewis cell lines [36]. In another study, two other saponins, oleiferasaponin $C_4$ and oleiferasaponin $C_5$ were tested against BEL-7402, BGC-823, MCF-7, HL-60 and KB and the extracted obtained from the whole plant of *Camellia oleifera* [37].

*Rumex hastatus* is one of the plant species that has shown some good results against cancer in addition to those previously mentioned. The crude saponins were effective and showed 93.3% tumor inhibition at 1000 μg/mL with $IC_{50}$ value of 18.1 μg/mL. Furthermore, it presented anti-angiogenic activity exhibiting 78.9% ($IC_{50}$ = 64.9 μg/mL) at 1000 μg/mL [38].

Three saponins known as 3β,24-dihydroxy-22β,30-epoxy-30-oxoolean-12-en-3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-xylopyranosyl-(1→2)-β-D-glucuronopyranoside, 3β,24-dihydroxy-22β,30-epoxy-30-oxoolean-12-en-3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-(3''-*O*-formyl)-galactopyranosyl-(1→2)-β-D-glucuronopyranoside and 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranoside punicanolic acid were obtained from green soya beans. Starting with 5.0 kg of fresh soya beans, 31 mg, 8.1 mg, and 1.2 mg, respectively, of the saponins were obtained after following various procedures. These saponins showed pharmacological activity but this time it was an anti-inflammatory action, with $IC_{50}$ values of 18.8, 16.1, and 13.2 μM, respectively, through the nitric oxide inhibition assay in LPS-stimulated RAW264.7 cells [39].

Another three triterpenic saponins demonstrated similar activity. The entagenic acid 28-*O*-[3-*O*-(2E,6R)-2,6-dimethyl-6-hydroxy-2,7-octadienoyl]-β-D-glucopyranosyl-(1→4)-[β-D-xylopyranosyl-(1→2)]-β-D-glucopyranosyl ester, the entagenic acid 28-*O*-[3-*O*-(2E,6R)-2,6-dimethyl-6-hydroxy-2,7-octadienoyl]-β-D-glucopyranosyl-(1→4)-[β-D-xylopyranosyl-(1→2)]-(6-*O*-acetyl)-β-D-glucopyranosyl ester, and 3β-*O*-2-acetamido-2-deoxy-β-D-glucopyranosyl entagenic acid 28-*O*-[2-*O*-(2E,6R)-2,6-dimethyl-6-hydroxy-2,7-octadienoyl]-β-D-glucopyranosyl-(1→4)-[β-D-xylopyranosyl-(1→2)]-(6-*O*-acetyl)-β-D-glucopyranosyl ester were isolated from 9 kg of the stems of *Entada phaseoloides.* These saponins yielded 13.0 mg, 5.7 mg, and 15.7 mg, respectively, and they reduced the production of nitric oxide in LPS-induced RAW264.7 cells at the concentrations of 15, 30, and 60 μM, indicating their anti-inflammatory activity [40].

Spirostanol saponins isolated from the rhizome of *Tupistra chinensis* are effective against five human cancer cell lines. In addition, they inhibited the nitric oxide production with $IC_{50}$ values of between 3.1 and 4.4 μM, showing, again, anti-inflammatory activity. These substances were elucidated as (25R)-5-β-spirostan-1β,3β-diol-1-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-xylopyranosido-3-*O*-α-L-rhamnopyranoside, (25S)-5β-spirostan-1β,3β-diol-1-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-xylopyranosido-3-O-α-L-rhamnopyranoside and (25R)-5ß-spirostan-1β,3β-diol-1-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-

xylopyranosido-3-*O*-β-D-glucopyranoside, and 5β-spirost-25(27)-en-1β,3β-diol-1-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-xylopyranosido-3-*O*-β-D-glucopyranoside [41].

Crude saponin fractions obtained from 2.6 kg of the rhizomes of *Anemone flaccida* yielded 83 g and were used to test the anti-arthritic effect on type 2 collagen-induced arthritis in rats. It decreased the pro-inflammatory cytokine levels in the type II collagen-induced rat model and in the LPS-induced RAW264.7 cells. The saponin enriched fraction contained flaccidoside II (28.1%), glycoside St-I4a (8.9%), and hederasaponin B (5.6%) [42].

A sample with more than 90% of saponins extracted from *Aralia taibaiensis* reduces myocardial injury in vitro and in vivo by activating the AMPK pathway. Pretreatment shows its efficacy in reducing infarct size, decreasing the levels of lactate dehydrogenase and creatine kinase and the blocking of apoptosis [43].

Three saponins known as tomentoside A, huzhangoside D, and clematoside were isolated using 4 kg of the roots and rhizomes of *Clematis graveolens* resulting in yields of 30 mg, 65 mg, and 30 mg, respectively. Tomentoside A was the most effective against *Aphis craccivora* and *Coptotermis homii*, followed by clematoside in the first case and by huzhangoside in the second case. This result shows their insecticidal activities [44]. Tomentoside A made from 100 g from tomato seeds was also cited in a study. The results showed this compound has potential anti-hyperglycemic power by regulating the intestinal glucose transport [45].

Saponins have also been related to anti-obesity due to their activity as pancreatic lipase inhibitors. One study was made with the *Cucumis sativus* fruit mesocarp. After various procedures, 1.498 g was obtained and this extract was used in comparison with other extracts. The results showed that all the samples inhibited the pancreatic lipase; however, the isolated saponins had the greatest inhibition and were more effective that Orlistat®, a drug used to treat obesity [46].

The neuroprotective effects of some saponins can be shown by tests with *Aralia elata*. The compound 3-*O*-[β-D-glucopyranosyl(1→2)-D-glucopyranosyl]-caulophyllogenin-28-*O*-β-D-glucopyranosyl ester was the most potent at 50 or 100 μM against $H_2O_2$-induced oxidative damage in SH-SY5Y cells (neuroblastoma cells) [47].

The saponin extract from *Quillaja brasiliensis* leaves was tested as an adjuvant in a bovine viral diarrhea virus vaccine in mice and it showed the capacity of stimulating cellular and humoral immune responses when it was used as an adjuvant [48]. Possibly, it can be useful in other approaches and future tests as an adjuvant for humans.

## 4.2 Antiprotozoal Activity of Saponins

### 4.2.1 Protozoal Diseases

The main biological effects described for saponins are related to activity against protozoa that are etiological agents of diseases such as trichomoniasis, leishmaniasis, sleeping sickness, and malaria.

Trichomoniasis is caused by *Trichomonas vaginalis*, a parasitic protozoan that occurs in the urogenital tract. This non-viral sexually transmitted disease, which is very widespread in humans, has an alarming number of people infected worldwide; there are about 276 million new cases per year. In addition, this infection increases susceptibility to HIV infection as well as the risk of cervical and prostate cancer [49].

Among the neglected diseases, sleeping sickness, or human African trypanosomiasis, is one of the most important diseases in public health. It is endemic in 36 African countries, threatening more than 60 million people in sub-Saharan Africa. *Trypanosoma brucei*, the etiological agent of sleeping sickness, is found in the bloodstream and may eventually settle in the brain. If left untreated, it causes coma and death [50]. The drugs available for the control and treatment of this disease are few in number; in fact, they are only four. In addition to the reduced number of therapeutic options, the side effects of these drugs are considerable.

Another trypanosomiasis, Chagas disease or American trypanosomiasis is an infectious disease, endemic in the American continent, caused by *Trypanosoma cruzi*. The protozoan has triatomines as its vectors; however, oral transmission may occur through the ingestion of contaminated food. The disease affects the heart and the gastrointestinal tract. There are about 12 million people with the chronic disease in the Americas, of which one million are in Brazil [51]. This neglected disease also has few treatment options; benznidazole and nifurtimox are the drugs used.

Malaria is an endemic disease transmitted by insects of the genus *Anopheles* contaminated by parasites of the genus *Plasmodium*. People living in tropical regions are the most affected. Two billion people are exposed to malaria and there are about a million deaths per year. According to WHO, the therapeutic arsenal has around 15 substances in current use, and frequently the parasite becomes resistance to those substances [52].

Leishmaniasis is another neglected tropical disease of importance in public health, caused by protozoa belonging to the genus *Leishmania* and it can be fatal if not treated. Depending on the *Leishmania* species and the host immune response, the infection may lead to the integumentary or visceral manifestation of the disease. Integumentary leishmaniasis includes cutaneous, mucocutaneous, and diffuse manifestations, with a negative impact on patients forced to live with skin lesions, which can lead to disfigurement and consequently social stigmatization [53, 54]. The World Health Organization estimates that there are 1.3 million new cases per year, of which 300,000 are visceral leishmaniasis (90% of them occur in Bangladesh, India, Brazil, Ethiopia, Nepal, and Sudan) and one million are cutaneous

leishmaniasis (Afghanistan, Algeria, Brazil, Colombia, Iran, Peru, Saudi Arabia, Sudan, and Syria) and mucocutaneous (Brazil, Peru, and Bolivia). The chemotherapeutic agents currently used in the treatment of visceral leishmaniasis and integumentary leishmaniasis, such as sodium stibogluconate, N-methyl-glucamine, pentamidine, and amphotericin B, lack activity when administered orally and require parenteral administration for long periods [55]. In addition, such chemotherapeutic agents are expensive and promote serious side effects due to high toxicity [55, 56].

### 4.2.2 Antiprotozoal Activity of Saponin Extracts

Antitrypanosomal Activity

The methanol extract of *Hyacinthoides non-scripta* exhibits in vitro antitrypanosomal activity. In a bioactivity-guided approach carried out with the methanol extracts obtained by the maceration of bulbs, leaves, scapes, shoots, and flowers of *H. non-scripta* against the *Trypanosoma brucei brucei* strain TC 221, only the extracts from leaves, shoots, and flowers are considered active. The most active extract is from the flower samples collected in May, which exhibits 99.1% of growth inhibition and has an $IC_{50}$ of 11.08 μg/mL. This antitrypanosomal activity of the methanol extract of *H. non-scripta* is attributed to the presence of saponin glycosides [57].

The hydroalcohol extract of *Pfaffia glomerata* roots and its fractions obtained by acid hydrolysis followed by solvent partitions are active against the tripomastigotes of the Y strain of *Trypanosoma cruzi.* The hydroalcoholic extract, rich in pfaffosides, is considered to have low activity, with an $IC_{50}$ of 181.69 μg/mL. However, the hexane fraction, rich in steroids and fatty acid esters, shows better activity, with an $IC_{50}$ of 47.89 μg/mL. Thus, the antitrypanosomal activity was not attributed to the presence of saponins in the extract [30].

The ethanol extract from leaves of *Carica papaya* has in vivo activity against *Trypanosoma evansi* (Steel 1885) in infected mice at doses of 75 mg/kg. This extract orally administered at 300 mg/kg for 3 days reduces by 82.2% and 87.8% the parasitemia in liver and kidney by the fourth day, respectively. Thus, the species *C. papaya*, besides its nutritional benefits, its leaf extract decreases the breeding ability of *T. evansi* [32].

Antitrichomonas Activity

The hydroalcohol extract of *Manilkara rufula* leaves and its rich saponin fraction have potent in vitro activity against trophozoites of *Trichomonas vaginalis* ATCC 30236 and eight fresh-clinical isolates. The saponin-rich fraction shows a MIC of 0.5 mg/mL against the ATCC 30236 strain. At the same concentration, the saponin fraction is effective against fresh-clinical isolates, causing a growth reduction of at least 85%. Furthermore, a synergistic effect with metronidazole at a sub-lethal

concentration (0.0026 mg/mL) is observed, including activity against the metronidazole-resistant isolates. The saponin-rich fraction is not cytotoxic to human vaginal epithelial line and HeLa cells at 0.5 mg/mL after 24 h in in vitro viability tests, but it is cytotoxic to HeLa cells after 48 h. The antitrichomonas activity involves damage to the parasite membrane; this has been confirmed by the analyses of ultrastructural changes, and the mechanism of action is not related to the immunomodulation of reactive oxygen species production [49].

### Antileishmanial Activity

In an in vitro antileishmanial study of a methanol extract and its fractions from the aerial parts of *Glinus oppositifolius*, the n-butanol fraction showed promising antileishmanial activity. At 50 μg/mL, this fraction increases pro-inflammatory cytokines and extracellular nitric oxide (NO) production from macrophages, which suggests antileishmanial activity based on the modulating pro- and anti-inflammatory cytokines and impairing the release of the reactive oxygen species (ROS) and NO [18]. This n-butanol fraction, after subjection to chromatography using Diaion HP 20 as the stationary phase gives a lead fraction when eluted with 50% methanol. The lead fraction is able to increase the extracellular NO five times when tested at 30 μg/mL, to promote the macrophage survival (81.5% viability at 50 μg/mL), and it has 50% cytotoxicity ($CC_{50}$) to macrophages above 100 μg/mL, suggesting it is an immunostimulatory agent with antileishmanial activity [18].

Commercially saponin extract (Sigma®), containing from 8 to 25% of sapogenin, probably obtained from the barks of *Quillaja* sp., has an anti-parasitic dose-response effect against promastigote and amastigote forms of *Leishmania major* strain IDUB/KE/83 after 24 h. In vitro assays against promastigotes indicate an $IC_{50}$ value of 24 μg/mL and an $IC_{90}$ value of 70 μg/mL, while against amastigotes, the $IC_{50}$ is 80 μg/mL and $IC_{90}$ is 280 μg/mL. The activity of the saponin extract is at least five-fold more effective than pentostam, the positive drug control. The $CC_{50}$ against mammalian cells is 3,400 μg/mL. These results suggest antileishmanial properties without toxicity for mammalian cells [31].

The in vitro antileishmanial evaluation of the extracts of *Ilex laurina* leaves obtained by percolation indicates promising effects of the ethyl acetate and dichloromethane extracts. The in vitro assays against *Leishmania panamensis* MHOM/CO/87/UA140epirGFP exhibits an effective concentration 50% ($EC_{50}$) of 7.5 μg/mL and an $EC_{50}$ of 12.3 μg/mL against intracellular amastigotes for the ethyl acetate and dichloromethane extracts, respectively. In assays against axenic amastigotes, ethyl acetate shows an $EC_{50}$ of 52.8 μg/mL, while dichloromethane exhibits an $EC_{50}$ of 20.3 μg/mL. Despite the moderate activity, high cytotoxicity is observed for both extracts against the human promonocytic cell line U937, with an $LC_{50}$ of 57.7 μg/mL and 17.0 μg/mL for the ethyl acetate and dichloromethane extracts, respectively. Amphotericin B, a reference drug, has better activity, with an $EC_{50}$ of 0.06 μg/mL for axenic amastigotes and 0.04 μg/mL for intracellular amastigotes, but the cytotoxicity is also high for U937 cells ($LC_{50} = 26.6$ μg/mL).

Another reference drug, pentavalent antimonial meglumine antimoniate, is only active against the intracellular amastigotes ($EC_{50} = 6.3$ μg/mL), with low cytotoxicity for U937 cells ($LC_{50} = 459.5$ μg/mL). Faced with the lower cytotoxicity of the ethyl acetate extract, this extract is considered more promising for further studies [23].

The hydroethanol extract from fruit pericarps of *Sapindus saponaria* obtained by maceration has antiproliferative effects against the promastigote and intracellular amastigote forms of *Leishmania amazonensis* MHOM/BR/75/Josefa. The $IC_{50}$ value for intracellular amastigotes is 181 μg/mL, and for promastigotes is 153.7 μg/mL, with a $CC_{50}$ of 81.66 μg/mL. A saponin-rich fraction obtained by solid-phase extraction in an octadecylsilane cartridge shows an $IC_{50}$ for intracellular amastigotes of 13.98 μg/mL, while the $IC_{50}$ for promastigotes is 25.41 μg/mL. Despite the best result for the saponin extract in the antileishmanial assays, the cytotoxicity for macrophages in the viability test is too high, 2.0 μg/mL, and with a high hemolytic effect. Due to the high toxicity, this fraction is not considered suitable for further studies related to antileishmanial use [58].

### 4.2.3 Antiprotozoal Activity of Isolated Saponins

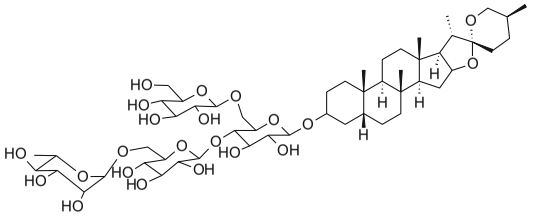
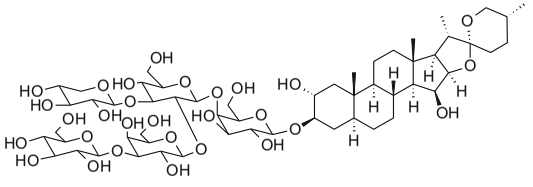
Different saponins have been tested for antiprotozoal activity and have been shown to be potent pharmacological targets against parasites. Among these isolated saponins, the small amount of the saponin necessary to inhibit different kinds of parasites is notable. Table 3 lists important antiprotozoal saponins of recent studies and other important saponins cited by recent reviews (2015–2019).

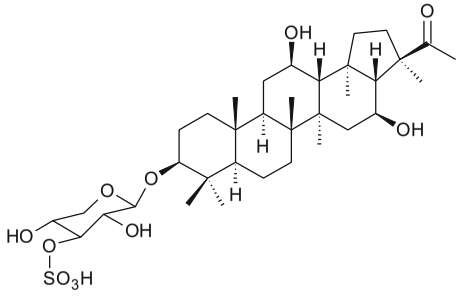
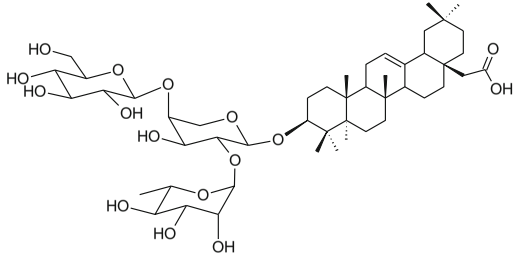
Figure 2 shows the graphical representation of the in vitro antiprotozoal activities of the most active saponins (all with an $IC_{50} < 30$ μg/mL) present in Table 3. Most of them (6) show considerable activity against *T. brucei brucei*, four of them obtained from *Mussaenda luteola*. Six saponins have high activity against the genus *Leishmania* with emphasis on the *L. infantum* strains that are very sensitive to hederacolchiside A1 (0.048 μg/mL), isolated from *Hedera colchica* and the saponins maesabalide III and IV, with an $IC_{50}$ lower than 0.1 μg/mL. Also noteworthy are the saponins active against *Plasmodium falciparum*, maesargentoside I and II, maesasaponin V3 and VI2, all of which exhibited $IC_{50}$ values lower than 10 μg/mL. The saponins that were tested against *Trichomonas vaginalis* were considered to have weak activity and therefore were not represented in Fig. 2. The most active saponin was the mixture S1 and S2 with an $IC_{50}$ value of 78 μg/mL.

## 5 General Considerations

The focus of this overview was to summarize the different properties linked to the saponins, particularly their antiprotozoal activity. The pharmacological activities of saponins have been exposed in multiple studies, showing their importance and the

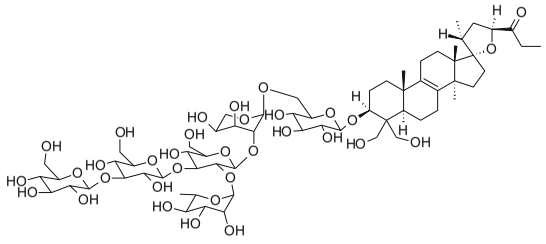
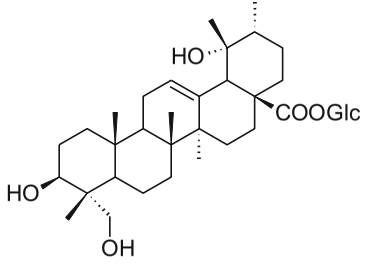
**Table 3** Isolated saponins with antiprotozoal activity

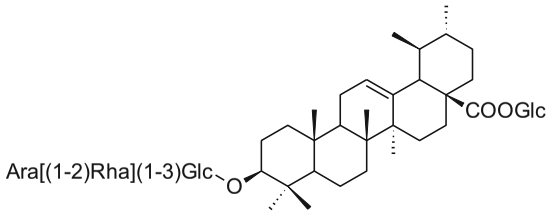
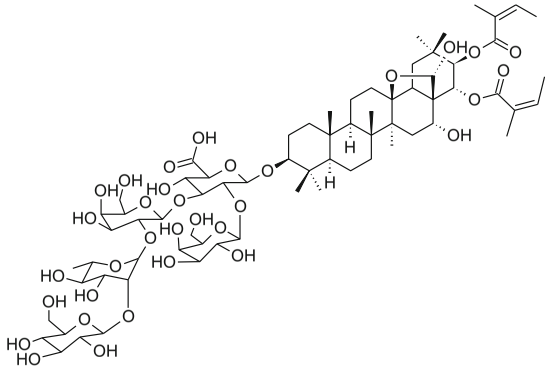
| Plant source | Saponin | Parasite | Ref. |
|---|---|---|---|
| *Asparagus racemosus*<br>(fruits)<br>Liliaceae<br>Methanol extract |  <br>1: Racemoside A | *Leishmania donovani*<br>promastigotes<br>$IC_{50} = 1.31$ μg/mL<br>*Leishmania donovani*<br>amastigotes $IC_{50} = 0.157$ μg/mL | [7–9, 59] |
| *Digitalis purpurea* (seeds)<br>Scrophulariaceae<br>Commercial |  <br>2: Digitonin | *Trypanosoma brucei brucei*<br>$IC_{50} = 8.47$ μg/mL | [60] |
| *Glinus oppositifolius*<br>(aerial parts) Molluginaceae<br>Butanol fraction from the methanol extract | | *Leishmania donovani*, intracellular parasites<br>$IC_{50} = 30.0$ μg/mL | [18] |

| | | | |
|---|---|---|---|
| |  <br>3: Spergulin A | | |
| *Hedera colchica* (leaves)<br>Araliaceae<br>Ethanol/water extract |  <br>4: Hederacolchiside $A_1$ | *Leishmania infantum* amastigotes<br>$IC_{50} = 0.048$ μg/mL | [9, 19, 61] |
| *Hyacinthoides non-scripta*<br>(flowers)<br>Asparagaceae<br>Methanol extract | | *Trypanosoma brucei brucei*<br>$IC_{50} = 28.0$ μg/mL | [57] |

(continued)

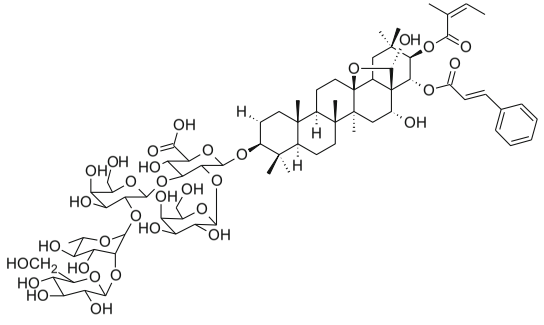
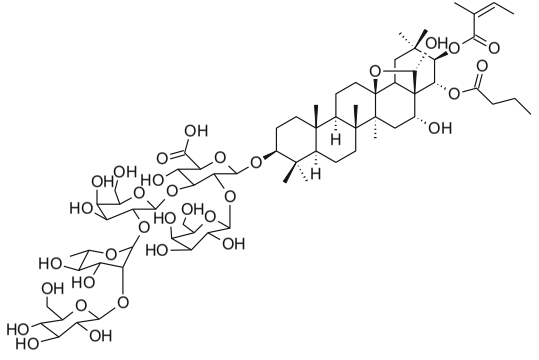
**Table 3** (continued)

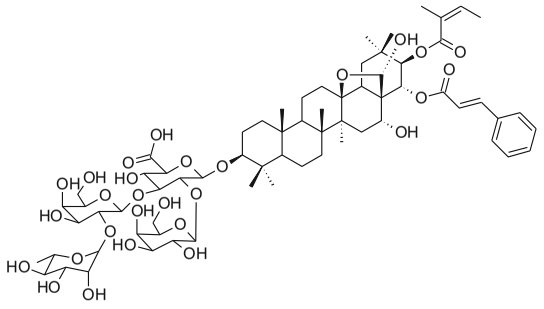
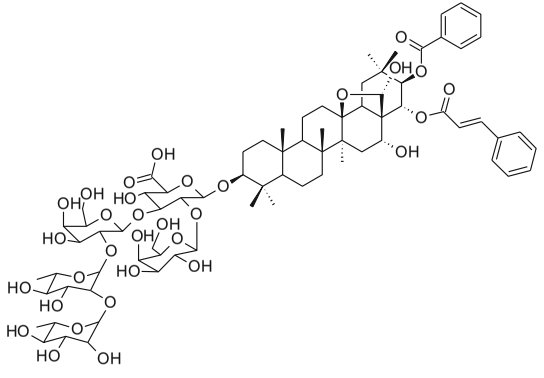
| Plant source | Saponin | Parasite | Ref. |
|---|---|---|---|
| |  <br>5: 3β-(*O*-β-D-glucopyranosyl-(1→3)-*O*-β-D-glucopyranosyl-(1→3)-[α-L-rhamnopyranosyl-(1→2)]-*O*-β-D-glucopyranosyl-(1→2)-*O*-α-L-arabinopyranosyl-(1→6)-*O*-β-D-glucopyranosyl) oxy-17,23-epoxy-28,29-dihydroxy-27-norlanost-8-em-24-one | | |
| *Ilex laurina* (leaves) Aquifoliaceae<br>Ethyl acetate extract (after other solvents) |  <br>6: Rotungenoside | *Leishmania (V.) panamensis*<br>$IC_{50} = 41.6$ µg/mL<br>(axenic amastigotes)<br>$IC_{50} = 5.9$ µg/mL (intracellular amastigotes) | [23] |
| *Ilex paraguariensis* (leaves)<br>Aquifoliaceae<br>Butanol extract | | *Trichomonas vaginalis*<br>$IC_{50} = 500$ µg/mL | [62] |

| | | | |
|---|---|---|---|
| |  <br>7: Metasaponin 2 | | |
| *Maesa argentea*<br>Leaves<br>(Myrsinaceae)<br>Methanol-water extract |  <br>8: Maesargentoside II | *Plasmodium falciparum*<br>$IC_{50} = 7.65$ µg/mL | [24] |
| *Maesa argentea*<br>(leaves)<br>Myrsinaceae<br>Methanol-water extract | | *Plasmodium falciparum*<br>$IC_{50} = 1.15$ µg/mL | [24] |

(continued)

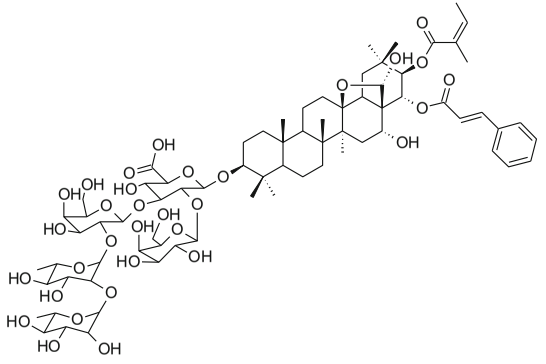
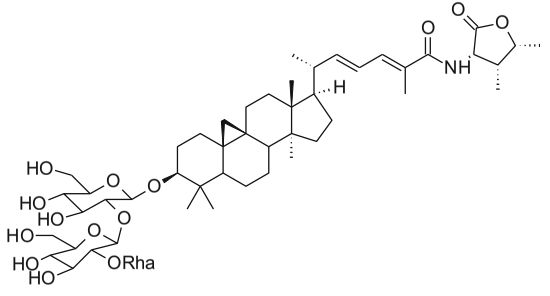
**Table 3** (continued)

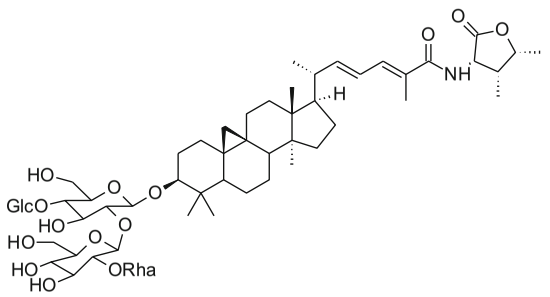
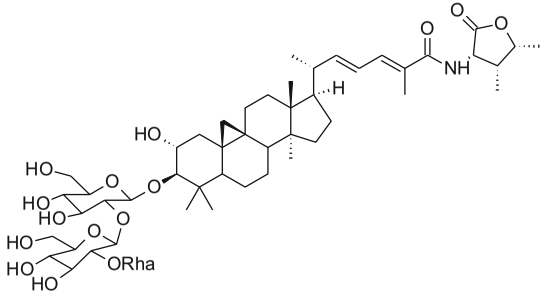
| Plant source | Saponin | Parasite | Ref. |
|---|---|---|---|
| |  9: Maesasaponin $V_3$ | | |
| *Maesa argentea* (leaves)<br>Myrsinaceae<br>Methanol-water extract |  10: Maesargentoside I | *Plasmodium falciparum*<br>$IC_{50} = 3.77$ µg/mL | [24] |

| | | | |
|---|---|---|---|
| *Maesa argentea* (leaves)<br>Myrsinaceae<br>Methanol-water extract |  <br>11: Maesasaponin $VI_2$ | *Plasmodium falciparum*<br>$IC_{50} = 1.96$ μg/mL | [24] |
| *Maesa balansae* (leaves)<br>Myrsinaceae<br>Butanol fraction from the methanol extract (after other solvents) |  <br>12: Maesabalide III | *Leishmania infantum*<br>$IC_{50} = 0.007$ μg/mL | [7, 28] |
| *Maesa balansae* (leaves) Myrsinaceae<br>Butanol fraction from the methanol extract (after other solvents) | | *Leishmania infantum*<br>$IC_{50} = 0.014$ μg/mL | [7, 28] |

(continued)

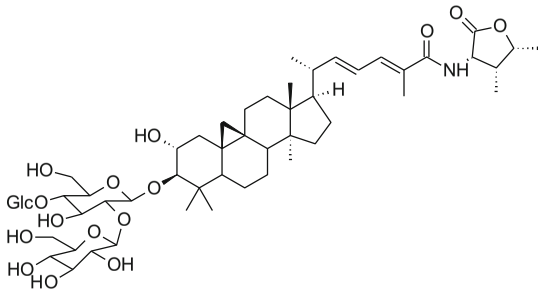
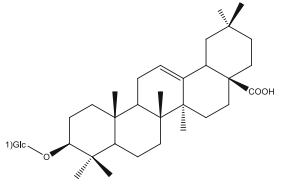
**Table 3** (continued)

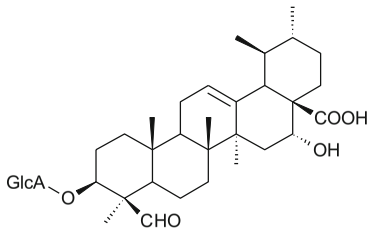
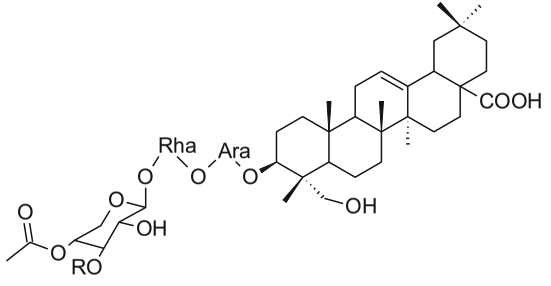
| Plant source | Saponin | Parasite | Ref. |
|---|---|---|---|
| |  <br>13: Maesabalide IV | | |
| *Mussaenda luteola* (aerial parts) Rubiaceae<br>Methanol-water extract |  <br>14: Heinsiagenin A 3-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)]-β-D-glucopyranoside | *Trypanosoma brucei brucei*<br>$IC_{50} = 9.13$ μg/mL | [29] |

| | | | |
|---|---|---|---|
| *Mussaenda luteola* (aerial parts) Rubiaceae Methanol-water extract |  <br>15: Heinsiagenin A 3-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)]-[β-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside | *Trypanosoma brucei brucei*<br>$IC_{50} = 3.08$ μg/mL | [29] |
| *Mussaenda luteola* (aerial parts) Rubiaceae Methanol-water extract |  <br>16: 2α-hydroxyheinsiagenin A 3-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)]-β-D-glucopyranoside | *Trypanosoma brucei brucei*<br>$IC_{50} = 2.75$ μg/mL | [29] |
| *Mussaenda luteola* (aerial parts) Rubiaceae Methanol-water extract | | *Trypanosoma brucei brucei*<br>$IC_{50} = 3.04$ μg/mL | [29] |

(continued)

**Table 3** (continued)

| Plant source | Saponin | Parasite | Ref. |
|---|---|---|---|
| |  <br>17: 2α-hydroxyheinsiagenin A 3-*O*-[β-D-glucopyranosyl-(1→2)]-[β-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside | | |
| *Passiflora alata* (leaves) Passifloraceae<br>Ethanol extract |  <br>18: 3-*O*-β-D-glucopyranosyl-(1-2)-β-D-glucopyranosyl-oleanolic acid | *Trichomonas vaginalis*<br>$IC_{50} = 250$ μg/mL | [62] |

| | | | |
|---|---|---|---|
| *Quillaja saponaria* (leaves) Quillajaceae<br>Commercial |  <br><br>19: 3-*O*-β-D-glucuronopyranosyl quillaic acid | *Trichomonas vaginalis*<br>$IC_{50} = 250$ μg/mL | [62] |
| *Sapindus saponaria*<br>(dry pericarps of the fruits)<br>Sapindaceae<br>Water-ethanol and butanol extracts |  <br><br>20: Saponin S1, R = $COCH_3$<br>Hederagenin-3-*O*-(3,4-di-*O*-acetyl-β-D-xylopyranosyl)-(1–3)-α-L-rhamnopyranosyl-(1–2)-α-L-arabinopyranoside;<br>21: Saponin S2, R = H<br>Hederagenin-3-*O*-(4-*O*-acetyl-β-D-xylopyranosyl)-(1–3)-α-L-rhamnopyranosyl-(1–2)-α-L-arabinopyranoside | *Trichomonas vaginalis*<br>$IC_{50} = 78$ μg/mL<br>(saponin S1 + saponin S2) | [62–64] |

*Glc* glucose, *GlcA* Glucuronic Acid, *Ara* Arabinose, *Rha* Rhamnose

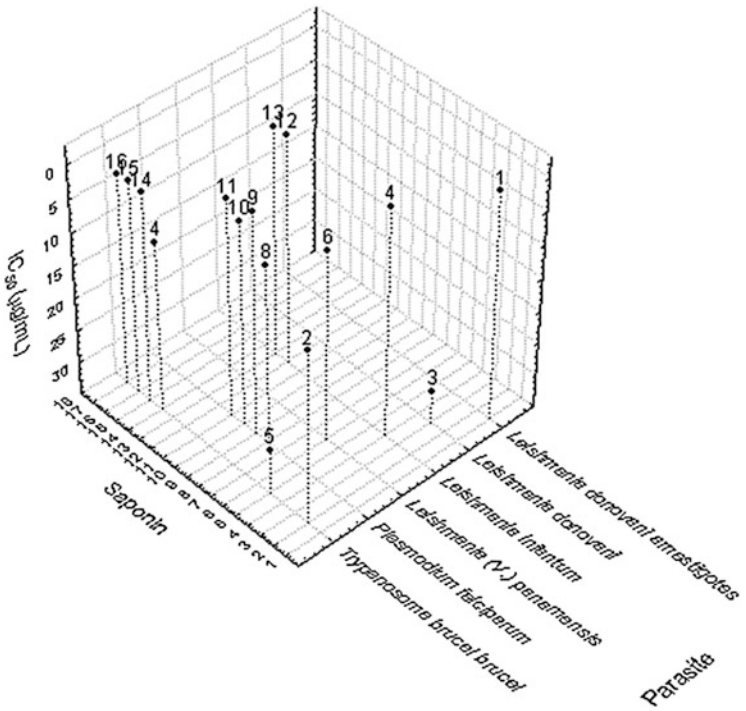



**Fig. 2** In vitro antiprotozoal activities of the most active saponins listed in Table 3, represented by the numbers in the Y-axis

reason why research groups need to keep on investing in this research line. Notable is the small amount of saponin necessary to inhibit different kinds of protozoans, showing their potent cytotoxic activity. Based on the responses in the different biological assays performed, more work should be conducted on saponins to define their medicinal properties as was done with this class of substances as vaccines.

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# Chagas Disease: Drug Development and Parasite Targets


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## Contents



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**Abstract** Chagas disease (CD) is a neglected disease that is endemic to Central and South America and is caused by the protozoan parasite *Trypanosoma cruzi*. The discovery of new drugs against CD has not made any significative progress, as the same two drugs have been in use since the 1960s. Benznidazole (BZN), the first-line treatment and Nifurtimox (NFX), the second-line treatment are both nitro-heterocyclic derivatives. Significant problems of resistance have emerged with both drugs. Although new drugs and new *Trypanosoma cruzi* targets are the focus of studies worldwide, their development and release onto the market remain unresolved. This chapter aims to review current drugs for Chagas disease and their targets, as well as to discuss the challenges that exist in the discovery of new drugs. Furthermore, the evidence that points to the need to strengthen a collaborative network between institutions is emphasized along with the importance of multi-omic studies to support the development of new drugs for Chagas disease.

**Keywords** Chagas disease, Drug discovery, Drug target, Omics platforms, *Trypanosoma cruzi*

# 1 Introduction

## *1.1 Chagas Disease: A Problem for Public Health*

Chagas disease (CD) from among the group of neglected tropical diseases (NTDs) is still an important disease worldwide with a high morbimortality of about 50,000 deaths each year. CD is endemic to Central and South American countries but currently, non-endemic places such as Canada, USA, Europe, Australia, and Japan have been affected due to the increasing global migration from endemic countries to non-endemic areas [1, 2]. The prevalence is variable depending on the location but is highest in Bolivia and Argentina. In the United States, it is estimated that more than 30,0000 Latin American immigrants are currently infected with Chagas disease [3, 4]. The major route is vector-based transmission, but other transmission routes have been found (Fig. 1), including sexual transmission. In summary, the spread of CD is related to the migration of individuals with Chagas disease to previously non-endemic countries [1, 5, 6].

CD is caused by infection with the protozoan *Trypanosoma cruzi* which has a complex life cycle with insect vectors of the subfamily Triatominae (Hemiptera: Reduviidae). *T. cruzi* diverged from other trypanosomatids about 200 million years ago; and it circulates in 120 species of mammals, including humans [7]. Historically, it has been established that Chagas' disease existed as early as 7050 B.C. Exhumed mummies from archaeological sites in both Peru and Chile showed positive for *T. cruzi*'s kinetoplast DNA by polymerase chain reaction (PCR) [5, 8]; Approximately seven million people are infected by *Trypanosoma cruzi*, and 1.8–2.4 million of these infected people will develop severe clinical manifestations [9, 10]. In its life cycle, the parasite presents two evolutive forms: the first is the circulating infective but not replicative form known as trypomastigotes, which is the longest phase, and

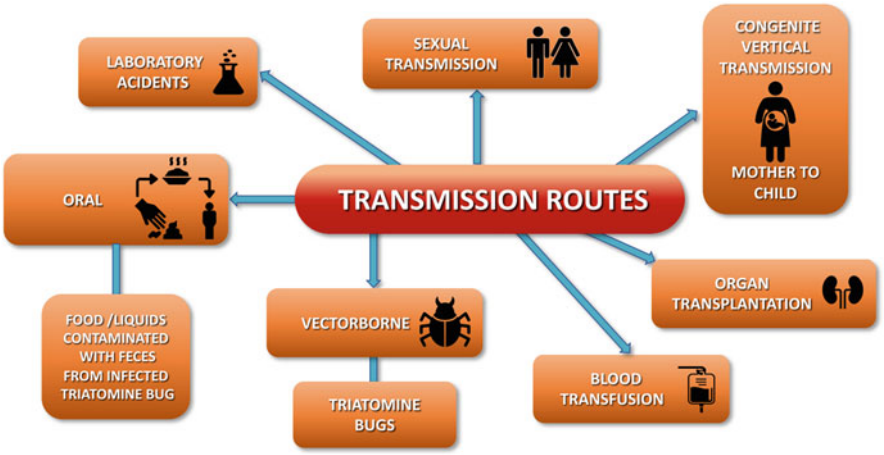



**Fig. 1** Main transmission routes for Chagas disease

the second is the replicative and intracellular forms, known as amastigotes. The extracellular amastigotes have also been shown to be infective [11]. The metacyclic trypomastigotes and epimastigotes forms are found only in the insect vector. The former is infective and although epimastigotes were considered non-infective, some differentiated epimastigotes may be infective to mammalian hosts [12]. Besides the complex life cycle there is a genetic polymorphism in the parasite populations showing multiple genotypes and phenotypes. Currently the species is subdivided into seven genetically discrete typing units (DTUs): TcI to TcVI and TcB, an additional clade associated with bats [13, 14]. *T. cruzi* I is the DTU with the broadest geographical distribution and associated with severe cardiomyopathies. Whole genome sequencing results of several TcI isolates and the genetic subdivisions within TcI may be needed in the future [15]. Different *Trypanosoma cruzi* strains have been isolated from patients and it has been suggested that these parasite strains, regardless of the clinical presentation, reflect the principal DTU circulating in a particular region. However in several orally transmitted outbreaks, sylvatic strains are implicated [13]. All these factors regarding this genotypic and the phenotypic differences of *T. cruzi* strains and the different geographic distribution of DTUs increase the complexity making standardization of serological tests [16].

There are two distinct phases in the infection: (1) The acute phase – after vector-borne *T. cruzi* exposure. The acute phase begins after an incubation period of 1–2 weeks, and is characterized by a high parasitemia and circulating trypomastigotes are detectable by microscopy in fresh blood. This stage could be asymptomatic (90%) or the patient could present symptoms (10%) such as fever, anorexia, and tachycardia. These symptoms disappear in 90% of the cases, the mortality rate of the acute phases is 5%. (2) The chronic phase – 8 to 12 weeks after infection, parasitemia levels become undetectable by microscopy and, in the absence of treatment, some infected patients progress to the chronic phase – 60–70% of the infected individuals do not develop symptoms and are identified as a group with the indeterminate form of the disease. Up to 30% of the chronically infected

people develop cardiac alterations and up to 10% develop digestive (megacolon or megaesophagus), neurological, or mixed alterations which may also require specific treatment [17]. The chronic chagasic cardiomyopathy is the most serious clinical manifestation of the disease [1, 2, 18]. The infection in an individual with immunodeficiency (HIV infection, organ transplantation, autoimmune disease, or oncological treatments) can be reactivated and generate great morbidity and mortality [19]. Another distinct difference between the two phases is that during the acute phase of CD the trypomastigotes are abundant in the peripheral blood, and during the chronic phase, the amastigotes are abundant in various tissues [12].

Recently in a study by McCall et al., the authors presented results that demonstrate that infection by *T. cruzi* modulates the fecal microbiome, suggesting host–microbe interaction in the CD [20]. In this work the authors applied metabolomics and sequencing of 16S rRNA during the acute and chronic stages of infection using a murine model of CD. Consequently, they were able to verify the microbial and chemical disturbances associated with the *T. cruzi* infection, highlighting the importance of multi-"omics" and poly-microbial studies in the area of parasitic diseases in general, and in CD in particular [20]. However, as there are no well-validated targets for CD, phenotypic screening of various compound collections is still considered the most useful and economical strategy to identify new leads or starting points [21].

At last, it is important to consider that Chagas disease and other neglected diseases are part of a group of illnesses for which a set of measures are being developed to prevent, control, and treat until the eradication of these diseases.

The World Health Organization established a current road map – WHO (2021–2030) coordinated by the public, private, the not-for-profit research Drugs for Neglected Disease Initiative (DNDi), and humanitarian medical organizations.

The Wellcome Trust, the Bill and Melinda Gates Foundation, and the NIH contribute with the research in this area, as well as world Research institutions, pharmaceutical companies such as GlaxoSmithKline, Tres Cantos Open Lab Foundation (TCOLF), Division of Biological Chemistry and Drug Discovery, School of Life Sciences, University of Dundee, The Novartis Institute for Tropical Disease (NITD), in cooperation with the Singapore Economic Development Board. All these efforts together bring a considerable development in the research of new drugs against these diseases [22–24].

## 2 Targets for the Treatment of Chagas Disease

Until this moment, there are no effective treatments against *Trypanosoma cruzi*. Currently, the research strategy is based on the biological differences between the parasite (*Trypanosoma cruzi*) and host cells (mammals) [25]. Intracellular parasites have high proteolytic activity, participating in various physiological and pathological events such as colonization, invasion, replication, differentiation, nutrition, dissemination, and evasion of the host's immune system [26–28].

In the last 20 years, new strategies and tools had been used in the field of drug discovery, including new computational methodologies for omics analysis and medicinal chemistry, screening of new drugs, the study of the relation of three-dimensional structures and functions of biological molecules [16, 29]. Furthermore, studies into the basic biochemistry of the parasite have identified metabolic pathways in *T. cruzi* that provide or could provide novel targets for chemotherapy [30].

The inhibition of specific enzymes, metabolic pathways, or organelles exclusive to the parasite is an interesting strategy, called *Target-Based Drug Discovery*. Proteasomes is one of the major targets not only for Chagas disease but also for Leishmaniasis and sleeping sickness caused *leishmania* spp. and *Trypanosoma brucei*, respectively [31].

Other enzymes such as α-carbonic anhydrases from *T. cruzi* have also been studied [16, 32]. Several essential pathways, such as the glycolysis, pentose phosphate, and the Redox metabolism present in *T. cruzi*, have also been identified. Studies of the isoprenoid pathway have resulted in drugs that are being used in clinical trials. The toxicity and selectivity of the protozoan organelles is another crucial factor, since few compounds show such features. The characteristics of acidocalcisomes make these organelles potential targets for trypanocidal drugs [30]. Some of these targets are abandoned and are no longer of interest.

Despite the increase and urgency of research for new trypanocidal compounds against the infectious forms of *T. cruzi,* there is still no effective cure for Chagas disease. Most of these compounds being researched end up as inadequate due to the high toxicity to host cells. The selection of the therapeutic target is all-important, as it favors the rational search for compounds that induce a specific therapeutic response against *T. cruzi* [16, 30, 33–35]. An improved differentiation of the potential targets in the parasites that are absent in humans will help fight this neglected disease. Numerous questions arise related to the research of new drugs, and some of them were reported by [16, 23]. One recognized problem is the lack of standard methodology, different strains, different detection methods, and biomarkers to evaluate responses to therapy, diagnosis, and monitoring drug efficacy. In addition, according to Chatelain [36] the absence of standardization of animal models designed for Chagas disease drug discovery is directly involved in the translational process failure. All these factors constitute barriers in the development of new drugs.

In the next section the most promising and consolidated *Trypanosoma cruzi* targets are discussed.

## *2.1 Drug Targets*

### 2.1.1 Cysteine Peptidase-Cruzipain

Some peptidases have been identified as possible targets for the development of new drugs, such as cysteine, serine, metallo- and threonine-peptidases. In general,

peptidases are hydrolytic enzymes that have the capacity to break the peptide bonds of proteins and protein fragments with selectivity and specificity [37, 38].

Among the peptidases of *T. cruzi*, we can highlight cruzipain, a cysteine peptidase, which has a prominent proteolytic activity in all the developmental stages of *T. cruzi.* Thus, cruzipain is a therapeutic target that has been intensely studied for the treatment of Chagas disease [39, 40]. Also, according to Cazzulo [39], recombinant enzymes have been developed, and different drugs are being studied that specifically inhibit cysteine peptidase in vitro, blocking the proliferation of epimastigotes, amastigotes, and metacyclogenesis of the parasite.

Cruzipain inhibitors cause the parasite to die in vitro, probably due to the accumulation of proteins within the Golgi complex, which results in an osmotic shock within the endoplasmic reticulum of the parasite [41]. This enzyme is present in organelles related to lysosomes and is associated with the plasma membrane [42, 43]. The K777 is a peptide derivative of vinyl sulfone that inhibits cruzipain irreversibly [44]. It is worth mentioning that many of these inhibitors have shown a lack of selectivity and low bioavailability in the in vivo assays. In addition, some compounds are potent cruzipain inhibitors but with slight effectiveness against *T. cruzi* in cell cultures. Concerted efforts are being made to overcome these difficulties and obtain an inhibitor with the selectivity and safety required to treat Chagas disease [44, 45]. This project to perform the preclinical studies demonstrating safety pharmacology and toxicology was started in 2010 by DNDI. Still, it was stopped in 2013 due to poor tolerability findings at low doses in primates and dogs [46]. Another derivative, the Neq0682, a reversible covalent inhibitor, of cysteine peptidases obtained by replacing the K777 vinyl sulfone group with a nitrile moiety was synthesized and is under study [47]. Other inhibitors against cruzipain are being studied such as quinoline [48], thiophen-2-iminothiazolidine derivatives, and thiophene–thiazolidine hybrids [49].

### 2.1.2 Kinetoplast Proteasome

The proteasome is an enzymatic complex formed by threonine peptidases. They are responsible for numerous biological functions in eukaryotic cells, such as the turnover of short-lived, abnormal/damaged proteins, cell cycle regulation, cell differentiation, signal transduction pathways, stress signaling, inflammatory responses, and apoptosis among others [50]. The 26S proteasome was identified in the epimastigote stage of Trypanosoma as a high molecular weight complex involved in parasite cell differentiation. This effect is mediated by the 20S proteasomal degradation of oxidized proteins through an ATP/ubiquitin-independent mechanism [51, 52]. In this context, it has been demonstrated that inhibition is associated with significant defects in parasite proliferation, turning on the proteasome as an attractive target for Chagas disease and other trypanosomatids and *Plasmodium falciparum* [53]. The Genomics Institute of the Novartis Research Foundation (GNF) has identified an azabenzoxazole compound series (GNF5343 and the optimized GNF6702) [54, 55]. In addition, GlaxoSmithKline (GSK) and

Dundee Drug Discovery have also identified a similar azabenzoxazole (GSK3494245). Azabenzoxazole (GNF6702), a non-competitive inhibitor of proteasome chymotrypsin-like activity, is a promising candidate for preclinical evaluation against neglected tropical diseases [22, 56]. This inhibitor was effective when tested against *Trypanosoma cruzi* as well as *T. brucei* and *Leishmania donovani*. Besides this, it does not inhibit the mammalian proteasome or growth of mammalian cells and is well tolerated in mice [16].

### 2.1.3 Carbonic Anhydrase

Carbonic anhydrases (CA, EC 4.2.1.1) are metalloenzymes with various physiological functions in all areas of life. They are involved in the pathological processes of human and prokaryotic/eukaryotic microorganisms such as bacteria, fungi, and protozoa. CAs catalyze the $CO_2$ reversible hydration: $CO_2 + H_2O \rightarrow HCO_3^- + H+$, which are involved in several physiological and pathological processes. $CO_2$ homeostasis, biosynthetic reactions, calcification, and tumorigenicity are some examples, among others. In addition, these enzymes are related to the growth and virulence of microbial pathogens. In researching new medicines, this target was established to develop anticonvulsants, anti-obesity, anticancer, and anti-infective drugs. In this context, CAs belonging to the α- and β-class were recently identified, cloned, and recognized their potential as new enzymatic targets in *T. cruzi* (αTcCA) and from *L. donovani chagasi* (LdccCA), respectively [57, 58].

In *Trypanosoma cruzi*, an α-CA was identified, cloned, and characterized by Pan et al. [58]. The α-TcCA has a high catalytic activity for the $CO_2$ hydration reaction. Inhibitors such as anions, sulfonamides, sulfamates, thiols, and hydroxamates were effective in low nanomolar in vitro tests [32, 59, 60]. One of the best inhibitors was hydroxamates which inhibited the growth of all three evolutive forms of the parasite at low concentrations (IC50 values from 7.0 μM to $<1$ μM) [61, 62]. Synthetic inhibitors such as sulfonamides have been tested against TcCA from *T. cruzi* with success. Bonardi et al. demonstrated that *N*-nitrosulfonamides and their salts inhibited the growth of the epimastigotes of *T. cruzi,* based on CA inhibition and are promising lead compounds for rational optimization of innovative agents for the treatment of Chagas disease [63]. All these results demonstrated the potential of the α-TcCA as a target yet underexplored for Chagas disease drugs [59]. The Cas is not a validated target for Chagas disease yet but is a possibility and further studies need to be performed to confirm this hypothesis.

### 2.1.4 Sirtuins

Silent-information regulator 2 (SIR2) proteins, or sirtuins, are a family of enzymes evolutionarily conserved and present in all kingdoms of life, from bacteria to higher eukaryotes [64]. According to Matutino Bastos et al., the inhibition of *T. cruzi* sirtuins by nicotinamide can cause growth arrest and morphologic alterations in

the parasite, thus being a possible candidate for a drug against CD [65]. In the search for new inhibitors, these same authors [65] characterized human sirtuin inhibitors against *T. cruzi* sirtuins. As a result, they reported seven inhibitors of sirtuins, where all compounds prevented the proliferation of *T. cruzi* in mammalian cells.

### 2.1.5 Cyclophilin

Cyclophilins are enzymes that perform several biological functions such as protein folding, where Cyclophilin D (CyPD) is a mitochondrial isoform with a crucial role in opening the pores for mitochondrial permeability [66]. It is known that this enzyme activity is inhibited by the immunosuppressant Cyclosporin A. In a study by Búa et al., the authors demonstrated anti-*T. cruzi* activity with Cyclosporin A through the inhibition of Cyclophilins, suggesting that this may be a molecular target [67]. According to Jha et al., Cyclophilin 19 is an enzyme present in all stages of life of *T. cruzi* participating in several functions, among them the generation of ROS that increases the growth of the parasite. In the study carried out by the authors, a mutant knock-out parasite of Cyp19 was generated, which was unable to replicate in cell cultures or in immuno-competent mice [68]. The authors also performed repeated inoculation of knock-out parasites where they observed specific antibodies and T-cell responses. According to the authors, these results demonstrate a 100% effective immunization in preventing Chagas disease. This study generated a patent entitled "Live attenuated parasitic vaccine" (US20200147148A1) [69].

### 2.1.6 *N*-myristoyltransferase

*N*-Myristoyltransferase (NMT, EC 2.3.1.97) is an enzyme that catalyzes the co- and posttranslational addition of myristic acid (C14: 0) onto the N-terminal glycine of specific proteins [70]. Studies have shown that NMT is both essential and druggable in *T. cruzi* [71], where it was shown that the inhibitor DDD8564630 caused a reduction in parasite proliferation in the epimastigote stage [72]. In a study by Herrera et al., the authors demonstrated the effectiveness of inhibitors as antiproliferative agents, presenting very low toxicity against mammalian cells. Where, according to the authors, it was possible to demonstrate its specificity and validation of NMT as a drug target using inhibitors with potential for future explorations such as anti-CD [73].

### 2.1.7 Pentose Phosphate Pathway: Glucose-6-Phosphate Dehydrogenase and Trypanothione Reductase

Glucose is metabolized through two major pathways: glycolytic and pentose phosphate (PPP). The PPP produces the ribose 5-phosphate (R5P) required for nucleotide synthesis and reducing power in the form of NADPH. One branch of the pathway is

the oxidative branch, involving glucose 6-phosphate dehydrogenase (G6PDH), among other enzymes. G6PDH is of central importance because it often has a high control coefficient for the PPP and can be considered a potential target for developing drugs for CD [74].

The studies with the PPP in Trypanosoma were scarce, and most of the pathway's enzymes, their properties, and subcellular localization were unknown until 2004 [74, 75]. PPP is important for parasites, considering that oxidative burst forming reactive oxygen is the first line of host cell defense against infection. Maugeri and coworkers [76] demonstrated an increased flow of the PPP pathway in *Trypanosoma cruzi* in response to oxidative stress. The parasite is highly sensitive to oxidative stress. The primary protection against reactive oxygen (ROS) is the Trypanothione, which is kept reduced by trypanothione reductase, using NADPH as a cofactor [75]. In addition to the cytosolic localization, PPP is present in the organelle glycosomes from trypanosomatids. Dehydroepiandrosterone (DHEA), epiandrosterone (EA), and derived 16/-bromoepiandrosterone (16BrEA) are known to be uncompetitive inhibitors of mammalian and trypanosome G6PDH [77]. Trypanothione reductase is an attractive target for antitrypanosomal drug. Some compounds such as Quinoxaline and Clomipramine showed activity against *T. cruzi* TR [78, 79] and quebrachamine, cephalotaxine, cryptolepine, tomatidine, solanidine, and solasodine were detected as potent inhibitors [80].

In a study by Fredo Naciuk et al. [81], the authors synthesized and evaluated 26 steroid derivatives of epiandrosterone (nonselective inhibitors of G6PDH) in enzymatic assays. As a result, compounds 40, 15, 39, and 6 showed a certain degree of selectivity in cell assays was achieved, at least in terms of toxicity in the system used (intracellular *T. cruzi* forms and rat cardiomyocytes). Although these results are promising, further studies are necessary to get more selective inhibitors, which increases the knowledge about the interactive action mechanisms of the inhibitors with the host cell/parasite.

Ortiz et al. [82] demonstrated in *Trypanosoma cruzi* using immunofluorescence a cytoplasmic distribution of G6PDH and the absence of signal in major organelles. In addition cytochemical assays confirmed that parasitic G6PDH is the molecular target of derivative epiandrosterone. These results together demonstrated that glucose 6-phosphate dehydrogenase shows a potential for a drug target for Chagas disease and other diseases caused by trypanosomatids as already shown for the African *Trypanosoma brucei* [82].

### 2.1.8 Ergosterol Biosynthesis Inhibitors

As ergosterol is essential for *T. cruzi*, enzyme inhibitors of its biosynthesis pathway have become common targets in studies [83]. Several studies have focused on the trypanosome ergosterol biosynthesis pathway (Fig. 2) due to the availability of existing drugs (posaconazole or ravuconazole) capable of inhibiting sterol 14 α-demethylase (CYP51). CYP51 is an essential enzyme for this pathway and

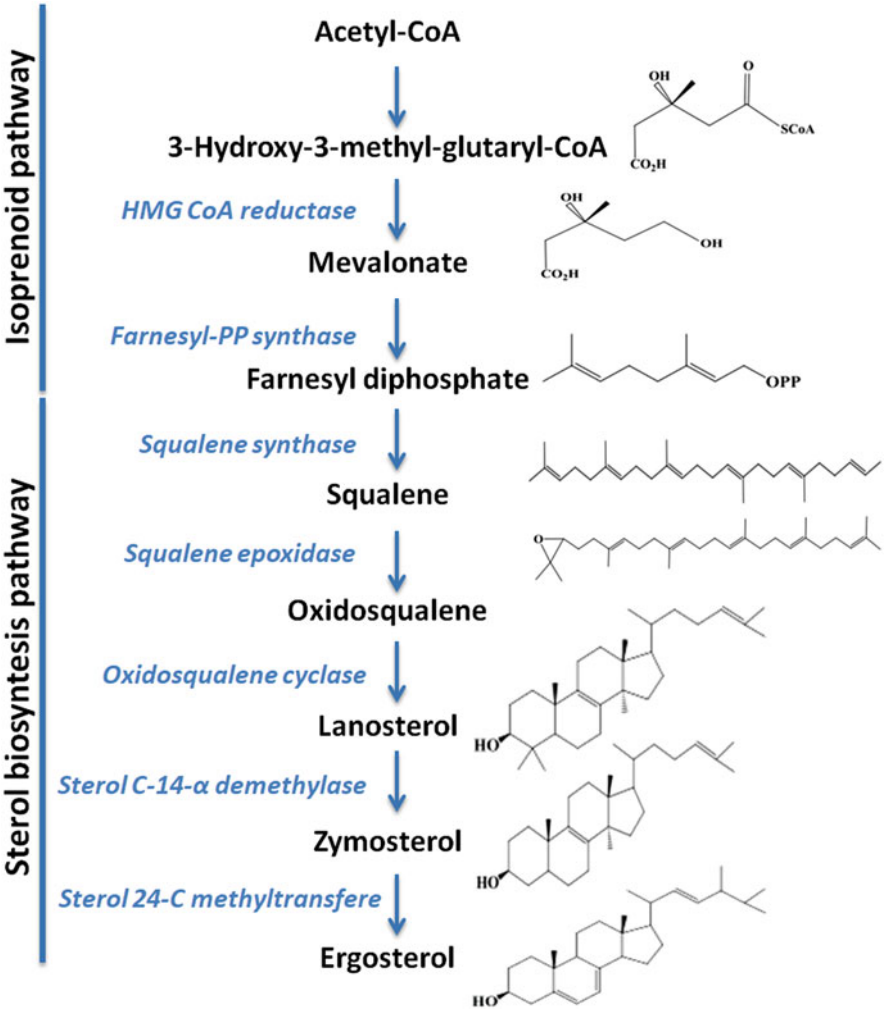



**Fig. 2** Simplified ergosterol biosynthesis pathway in *T. cruzi* epimastigotes. Critical enzymes (blue color) of the ergosterol pathway. Adapted from [84]

its inhibition reduces the capacity for invasion of heart cells by trypomastigotes and inhibits the multiplication of amastigotes [85, 86]. Posaconazole is not yet a viable alternative as it has a high cost and has a structure that is difficult to interact with *T. cruzi* CYP51 [86]. According to Osorio-Méndez and Cevallos [83], the evidence suggests that these inhibitors effectively fight infection in vitro and murine models; however, they have failed in clinical trials [23]. It is worth noting that the failure of CYP51 as a drug target is due to the cytostatic consequence of inhibition [23]. Consequently, assays have been developed to filter out compounds identified in phenotypic screens with this undesirable mode of action [87]. However, it may be possible

to rescue posaconazole by combination therapy described recently by Rocha-Hasler et al. [88] demonstrating that Tomatidine improves the potency of posaconazole as antitrypanosomal agent.

### 2.1.9 Sphingolipids

Sphingolipids are biological molecules found in eukaryotes and prokaryotes. Their structure is composed by a sphingoid base with a fatty acid attached through an amide bond, forming the ceramide. A variable polar head-group is present (phosphocholine, inositol phosphate, or carbohydrates). In epimastigotes from *Trypanosoma cruzi* glycosphingolipids were detected [89]. The parasite synthesizes inositol phosphorylceramide (IPC) and sphingomyelin (SM) and its expression is modulated during parasite development. The IPC synthase enzyme catalyzes the transfer of inositol phosphate to ceramide moiety. Several glycoconjugates are attached to membranes by a glycosylphosphatidylinositol (GPI) anchor and in this context *T. cruzi* have different surface molecules including the glycoinositolphospholipids (GIPLs, in epimastigotes), trans-sialidase and Tc-85 glycoprotein in trypomastigotes, mucins in epimastigotes and metacyclic forms and complex GPI-anchored glycopeptide called NETNES in epimastigotes. All these molecules have important biological function on parasite related to the antigenicity, pathogenesis, cellular survival, and programmed cell death (PCD) and in cellular survival [90–92].

Landoni et al. [93] studied the effect of tamoxifen (TAM) on the sphingolipid pathway of *T. cruzi*. TAM is an anti-estrogen used for the treatment of breast cancer. This drug is involved in apoptosis mechanisms. Although there are few studies about the effect of TAM in the sphingolipid pathway, the authors tested it in *Trypanosoma cruzi*. The results demonstrated a dose-dependent inhibition according to the evolutive stage of the parasite. Lipid extracts from epimastigotes were analyzed by MALDI-TOF and HPLC-ESI mass spectrometry. The results showed that after TAM treatment, a high and discrete increase in the level of ceramide/ceramide-1P and sphingosine, respectively, indicates the involvement of TAM in the breakdown of ceramide. Microscopy analysis and flow cytometry of the treated parasite demonstrated an apoptotic-like death process. Previous studies of Miguel et al. [94] showed that TAM was ineffective in the treatment of the acute phase of Chagas disease in mice. These results suggest that the sphingolipid pathway could be a target for drug development in Chagas disease. More studies are needed, including tests with new concentrations of the drug and evaluation of toxicity in host cells.

### 2.1.10 Intracellular Calcium Homeostasis

$Ca^{2+}$ is an essential signaling messenger in eukaryotic cells, including the *Trypanosoma cruzi* and other trypanosomatids [95–97]. The function of $Ca^{2+}$ as a signaling messenger in trypanosomatids is well documented in several biological

functions like flagellar movements, cellular differentiation, invasion of the host cell, osmoregulation, and nitric oxide transduction pathway [95, 98–108]. $Ca^{2+}$ concentration in *T. cruzi* is regulated by mechanisms present in the plasma membrane and by intracellular organelles such as mitochondria, endoplasmic reticulum, and acidocalcisomes. The maintenance of calcium homeostasis is very important considering that $Ca^{2+}$ is involved in the apoptotic process [109, 110]. In this context the disruption of intracellular calcium homeostasis has been proposed by Benaim et al. as a possible therapeutic target for drug development in Chagas disease [25]. The amiodarone and derivatives, for instance, an antiarrhythmic drug showed trypanocidal effects through disrupting the parasite $Ca^{2+}$.

### 2.1.11 Acidocalcisomes

Acidocalcisomes are organelles that play an important role in osmoregulation which are rich in polyphosphate bound to calcium and different cations such as magnesium, calcium, sodium, and zinc [111, 112]. The matrix contains enzymes related to poly P metabolism and the membrane of the acidocalcisomes has several pumps and transporters including a $Ca^{2+}$ -ATPase for $Ca^{2+}$ uptake. The $Ca^{2+}$ release is controlled by the inositol 1,4,5-trisphosphate receptor (IP3R) that is located in acidocalcisomes [95, 113]. Chiurillo et al. demonstrated that the remotion of the IP3R gene by CRISPR/Cas9 genome editing inhibits host cell invasion by trypomastigotes [114]. In contrast, TcIP3R overexpressing parasites showed decreased metacyclogenesis, trypomastigote host cell invasion, and intracellular amastigote replication. Summarizing $Ca^{2+}$ signaling is important for the *T cruzi* differentiation for host cell invasion and to maintain cellular bioenergetics [95, 114]. Based on these facts it had been suggested that the acidocalcisomes and their components could be potential targets for chemotherapy [115, 116].

### 2.1.12 Kinetoplast

Trypanosomes have a single mitochondria that has the peculiar characteristic of having a circular DNA network known as kinetoplast or kDNA [117, 118]. The kDNA is composed of circular and interconnected DNA molecules forming a single network, therefore, knowledge of this topological architecture is fundamental for understanding the replication and segregation of the kDNA circles [119]. In a study by Zuma et al. [120] the authors analyzed the effects of Berenil on the ultrastructure and replication of *T. cruzi* kDNA. As a result, the authors demonstrated that Berenil caused significant changes in the kDNA arrangement and a reduction in the growth of *T. cruzi*, but cell viability was not affected. The authors suggest that Berenil mainly affects kDNA topology and replication, demonstrating, according to them, that Kinetoplast represents a potential target against trypanosomatids [120].

According to Cavalcanti and de Souza [119], previous studies used atomic force microscopy to analyze the effect of acriflavine, an intercalating drug [121] in the

*T. cruzi* kDNA network. As a result, it was possible to evaluate the structure of kdna and investigate the topology changes caused by drugs, and the authors proposed that kdna can be an interesting target because it is affected by DNA-binding drugs, intercalating agents, and topoisomerase inhibitors [119].

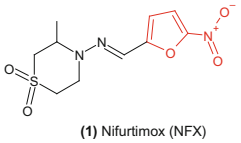
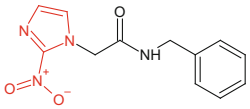
## 3 Current Treatments for Chagas Disease

Only two drugs have been used to treat CD, nifurtimox or (RS)-3-methyl-*N*-[(1E)-(5-nitro-2-furyl)methylene] thiomorpholin-4-amine 1,1-dioxide (NFX – Tables 1, 1) and benznidazole or *N*-benzyl-2-(2-nitro-1H-imidazol-1-yl)acetamide, which is a 2-nitroimidazole derivative (BZN – Tables 1, 2). Both were discovered more than 50 years ago, showing that there is a continued lack of investment in research and development (R&D). The justification for this is that the predominance of CD is in emerging countries; consequently, the pharmaceutical industries are not interested in investing in new antichagasic drugs and their excessive costs for research in developing countries [122].

NFX was the first drug developed for this purpose and has been used since 1965 and BZN since 1971. BZN was first produced by the pharmaceutical company Roche (Rochagan® and Radanil®), now it is manufactured by the Pharmaceutical Laboratory of the State of Pernambuco (Lafepe), Brazil, and by the private laboratory in Elea (Abarax®), Argentina. NFX is free of charge through a WHO-Bayer agreement [17, 123, 124].

Studies of the mechanisms of both drugs are not fully understood yet. NFX and BZN are prodrugs and require activation by enzymatic activity. Wilkinson and coworkers [125] proposed that nitroreductase is the main enzyme involved in the activation of nitro-heterocyclic drugs in *T. cruzi*. In general, nitroreductases reduce the nitro group present in both the nitro-heterocyclic compounds, generating

**Table 1** The main contraindications and indications for Benznidazole and Nifurtimox

| Drug | Contraindications | Indications |
|---|---|---|
|  **(1)** Nifurtimox (NFX) | The first trimester of pregnancy; Woman who are breastfeeding; Patients with a history of mental problems or seizures; Patients with a history of psychiatric or neurological disorders [122] | Congenitally infected newborns<br>Acute phase |
|  **(2)** Benznidazole (BZN) | Liver failure, kidney failure, pregnancy [17] | Acute phase<br>Congenitally infected newborns<br>Acute phase<br>Children under long-term therapy |

metabolites with different reactivities for each drug, such as molecules considered free radicals (e.g., R–NO-) and electrophilic metabolites (e.g., R–NHOH) [3, 126].

However, NFX and BZN require distinct enzymes for biological activation. For NFX, the proposed mechanism is through the one-electron route (type II nitroreductase). A re-oxidation leads to reactive oxygen species formation in cells (oxidative stress); the production of highly toxic oxygen metabolites and oxidative stress to the parasite by generating the nitro radical, redox cycling, and production of $O_2^-$ and $H_2O_2$ [3, 122, 127, 128]. In addition, some studies have proposed that the reduction of NFX by nitroreductase can open the furan ring producing an unsaturated open-chain nitrile that is as cytotoxic as the parent compound. This suggests that NFX trypanocidal activity does not necessarily involve oxidative stress [129, 130].

For the benznidazole the two-electron route (type I nitroreductase) in the mechanism forming reactive nitroso and hydroxylamine intermediates (chemical stress). The hydroxylamine derivative (R–NHOH) converts to glyoxal, a highly cytotoxic and mutagenic compound [129]. Some studies indicate that the reactive species generated in BZN metabolites can improve phagocytosis, increase trypanosome death through IFN-γ induction, and inhibit *T. cruzi* NADH fumarate reductase (thus) inducing mitochondrial DNA damage. The *T. cruzi* cytotoxic activity of these metabolites may involve covalent modification of macromolecules such as DNA, proteins, and lipids [131, 132].

The chemical species generated by these drugs are highly reactive and can affect other molecules, especially in the vertebrate host. Consequently, this low specificity of action on the parasite contributes to the cytotoxic effects observed in treatments with patients. *T. cruzi* is probably deficient in the metabolic detoxification mechanisms for oxygen and that makes it very susceptible to partial reduction products of oxygen, consequently, it is more sensitive to oxidation than the vertebrate cells [3, 127, 128].

NFX and BZN are almost 100% effective in curing CD if given soon after infection, at the onset of the acute phase, in cases of congenital transmission and for those in whom the infection has been reactivated due to immunosuppression. However, the efficacy of both reduces as time passes [17]. BZN is usually the first-line treatment in most countries because it has a better safety record and efficacy profile than Nifurtimox, mainly for adult patients that require prolonged administration [129]. In the acute and undetermined chronic phases, treatment with BZN has proven to be highly effective and about 80% of patients have no sign of parasites in the blood after 12 months of treatment. The other benefits of treatment with BZN are the high cure rates in infants with congenital infection and in children with chronic infection [122].

In 2011, a partnership between DNDi and Lafepe laboratory (Brazil) allowed the development of the first pediatric formulation of benznidazole for the treatment of children with CD. Following partnerships with Chemo Research, Exeltis USA, Mundo Sano Foundation and Laboratory ELEA PHOENIX were set up aiming to enable a broader registration in endemic countries, with a commitment to make the pediatric formulation more widely available. In 2017, the FDA granted approval for

the use of benznidazole in children with CD aged between 2 and 12 years old [17, 123, 133].

The treatment of the chronic phases of CD in adults with BZN on the growth rates of negative parasitemia increases with treatment time but there are no significant modifications with increased drug dosages or modifications in the drug formulation. Studies have demonstrated that negative parasitemia in patients with chronic CD has been observed after treatment from 55.97% after 2 months to 62.59% after 8–-16 months and 72.81% after 9–11 years [122, 123, 133, 134]. Although BZN is able to reduce serum parasite detection in chronic CD, it does not significantly reduce cardiac clinical deterioration [135, 136].

NFX treatment studies of 114 adults with chronic Chagas were conducted. These studies demonstrated positive serology after an average of 6.6 years for all of them; and *T. cruzi* was not detected in 93.9% of the cases after treatment. However, in these cases, according to the current cure criterion, it is not possible to interpret the obtained results as a parasitological cure [137]. In treatment with both drugs, BZN and NFX, therapeutic failures are common for reasons that include influences of the parasite and host genetics, and the effects of toxicity on adherence to the treatment. The long-term therapy increases the chances of NFX presenting more intense adverse effects than BZN. The adverse reactions are more frequently reported with older aged patients with both NFX and BZN. In general, NFX has shown higher toxicity and adverse effects than BZN, including increased oxidative stress in rat pancreas and heart [135].

Problems related to suffering from adverse drug reactions (ADRs) are reported in more than 30% of the patients treated with BZN, especially those in the chronic phase. These effects involve dermatitis, disorders in vision, myelosuppression, and peripheral polyneuropathy, as well as gastrointestinal system disorders [16, 122, 138, 139].

The main contraindications to treatment with BZN are liver or kidney failure and pregnancy. NFX is contraindicated in patients with a history of psychiatric or neurological disorders (Table 1) [140, 141].

Additionally, significant problems of resistance have emerged with both drugs [17, 123]. The mechanism of BZN and NFX resistance has been associated with the deletion of copies of genes encoding two different nitroreductases, namely old yellow enzyme (TcOYE) or prostaglandin synthase, and trypanosomal type I nitroreductase [122].

## 4 New Drugs and Clinical Trials

### *4.1 Drugs in Clinical Trials*

Since the introduction of pharmacotherapy for CD with BZN and NFX, only allopurinol and some azoles, such as: itraconazole, fluconazole, ketoconazole,

posaconazole (POSA), E1224/ravuconazole (RAV) and fexinidazole have been tested for the treatment of CD [3].

### 4.1.1 Fexinidazole: First Global Approval

Fexinidazole (FXN) is a promising broad-spectrum antiparasitic agent with clinical trials already taking place. FXN was first discovered by Sanofi (former Hoechst AG) and was identified by the DNDi in 2005 as having activity against *Trypanosoma brucei gambiense*, *T. b. rhodesiense,* and *T. cruzi* [142]. This compound has a nitro group in its composition that is metabolized by the parasite nitroreductases, forming reactive species that inhibits DNA synthesis [124].

Clinical studies with FXN have been performed with both CD and visceral leishmaniasis patients [143]. Dose-finding studies over a maximum treatment time of 8 weeks were conducted with chronic indeterminate Chagas disease patients. A Phase II Proof of Concept (PoC) study of fexinidazole was initiated in 2014 in Cochabamba and Tarija, Bolivia. However, the study was interrupted due to safety and tolerability problems. Further analysis of the results showed high efficacy findings were at the lowest dose tested for all treatment durations. The higher doses of the drug were considered unsafe after only 14 days. However, the clinical study follow-up was extended to 12 months [140]. FXN has demonstrated its therapeutic potential against CD, since it has been approved by the European Medicines Agency (EMA) for the treatment of African trypanosomiasis, in both adults and children [124, 142].

### 4.1.2 E1224/Ravuconazole

E1224 (fosravuconazole or fosravuconazole L-lysine ethanolate) is a prodrug of ravuconazole (RVZ), which is a potent inhibitor of ergosterol biosynthesis with activity against a wide range of fungal species [144–146]. RVZ is a potent CYP51 inhibitor, but studies have shown that it has a short terminal half-life, where in studies with murine models it presented limited in vivo activity due to unfavorable pharmacokinetic properties [147, 148]. In studies with canine models, the results were suppressive but not curative due to the short terminal half-life [147]. In a clinical study with humans led by DNDi, the drug E1224 presented a rapid, but transient, parasite clearance effect – suggesting that this drug has a static rather than a parasiticidal mechanism of action [145, 146]. It was also observed that the combination of RVZ with BZN did not significantly affect efficacy [144]. According to Spósito et al. [148] the authors suggest that therapeutic failure may be related to inadequate azole levels in tissues and suboptimal drug exposure [148].

### 4.1.3 New Drugs Discovery

The knowledge of parasite biology has, over the last decades, discovered small molecules that could be potential chemotherapeutic targets in the parasite for CD treatment, such as: enzymes that participate in the synthesis of ergosterol in *T. cruzi*; enzymes of the trypanothione metabolism such as trypanothione reductase; Cruzipain; Carbonic anhydrases; among others [84].

The potent activity of azole antifungal drugs against *T. cruzi* were reported more than 30 years ago. Since then, several azole antifungal drugs such as ketoconazole, fluconazole, itraconazole, posaconazole, D0870 and others, have been reported as sterol 14 α-demethylase inhibitors (CYP51) [149]. Triazole compounds such as posaconazole and D0870 have been shown to be effective at curing mice with chronic CD. On the other hand, clinical studies with ketoconazole or itraconazole in humans with chronic CD have not presented any significant cure of the disease. However, studies have been published demonstrating the synergistic activity of azole drugs with various other compounds, indicating that a combination of chemotherapies may be an effective strategy as this field moves ahead [149]. VNI is the first nonantifungal compound CYP51 inhibitor to target the 14 α-demethylase activity of *T. cruzi*. This compound demonstrated a cure for both the acute and chronic phases of infection with the Tulahuen *T. cruzi* strain in mice but failed to cure mice infected with the Y and Colombian *T. cruzi* strains in both phases of the infection [150].

Several compounds have been shown to inhibit *T. cruzi* trypanothione reductase and affect parasite growth in vitro or in vivo, such as nitrofurans and naphthoquinones, phenothiazines and crystal violet, diphenyl sulfide derivatives, polyamine derivatives, dibenzazepines, bisbenzylisoquinoline alkaloids, ajoene, acridines, terpyridine platinum complexes, and Mannich bases as well as some natural products [84, 151]. Buthionine sulfoximine (BSO) has been shown to be a potential drug candidate against *T. cruzi* alone, or jointly with free radical-producing drugs such as nitrofurazone and benzimidazole [84]. K777, a vinyl sulfone derivative, is a potentially irreversible inhibitor of cruzipain. Nevertheless, preclinical studies demonstrated poor tolerability even at low doses in primates and dogs. Novel scaffolds for the inhibition of cruzipain were identified from high-throughput screening (HTS) of GlaxoSmithKline HAT (Human African Trypanosomes) and Chagas chemical boxes [84].

Several aromatic/heterocyclic sulfonamides have been studied as carbonic anhydrases of *T. cruzi* (TcCA) inhibitors. The studies were performed against mammalian enzymes (hCA I and hCA II) and the inhibitors showed a greater inhibition of the *T. cruzi* TcCA indicating a positive selectivity [58]. Additionally, a series of the 4,5-dihydroisoxazoles incorporating hydroxamate moieties showed that they could also act as inhibitors of the *T. cruzi* α-CA and of peptidases from this pathogen, such as the cysteine and metallo-peptidase. These series were assayed in vitro against the tree forms of *T. cruzi* and in vivo. These studies showed that a leading compound had potential values for the growth inhibition of all three developmental forms of the Y strain of *T. cruzi* at relatively low concentrations [61].

## 5 Omics Platforms in Chagas Disease

Even after more than 100 years of the discovery of Chagas disease, and despite an enormous scientific research effort, this disease is still a major threat in several Latin American countries and an emerging global health problem [16, 152]. In work carried out by Alonso-Padilla et al. on strategies to diagnose and treat patients with CD, the authors cite the lack of public policies and funding as factors that make facing the problem even more challenging [10]. The authors attest that when there is no treatment ~30% of those chronically infected will develop cardiac and/or digestive disorders. According to the authors, a deeper understanding of the parasite's biology and its interactions with the host is of fundamental importance for the discovery of safer drugs or vaccines. Therefore, studies on the genomics of the parasite and the host combined with other omics are essential to determine the factors that lead to the development of CD [10]. Research into the biology of parasites requires a sophisticated and integrated computational platform that allows the analysis of large volumes of data [153].

Platforms with large amounts of omic data (proteomics, genomics, transcriptomics, metabolomics, etc.) have already been created and continue to be developed at an ever faster rate, but the databases have heterogeneous formats that are often difficult to integrate with experimental data [153]. According to Wooden et al., transcriptomic, genomic, and omic data (proteome, metabolome, kinome, methylome, acetylome, lipidome, microbiome, phenome, exposome, meta-genome, and interactome) have been increasingly deposited for public use, however, this excess of information has made the analysis of these omic data sets a challenging task [152].

In relation to CD, other types of genetic studies, such as transcriptomics and epigenetics, are needed to expand and integrate the genomic data already studied, in addition to the need to understand how environmental factors imply susceptibility to the disease [154]. According to Talavera-López and Andersson [155], integrative omic approaches will be able to treat, on a large scale, biological information from the most diverse areas such as drug resistance, epidemiology, genetic exchange, immune evasion mechanisms, genetic function, etc. However, according to these authors, additional work is also needed for a complete characterization of individual targets and families of genes, and yet other aspects of parasitic biology are still relatively unexplored. Therefore, they emphasize that a lot of basic research is needed before these issues can be effectively addressed [155]. In summary, Talavera-López and Andersson highlighted some points that make parasitic research problematic: (a) Sampling that is difficult to perform. When they are carried out, they are often not collected in a standardized manner, limited metadata and often there is no sampling strategy guided by a specific biological issue. (b) Requirement for field studies with visits to remote locations that implies complex and costly logistics. (c) Some parasites such as Leishmania, *T. cruzi, T. rangeli,* etc. can only grow in vitro using culture media and even cloning by limiting dilution. In this way, a level of uncertainty is introduced in the experiment, considering that the parasites can

change according to the culture medium in which they grow. And often the clones that dominate the culture may not be the same ones that occur in the host [155].

According to Sánchez-Ovejero et al., the integration of omic data is essential to understand the host–parasite relationship [156]. However, as demonstrated by Vermelho et al. [16], the technological advances to date have not resulted in the discovery of new drugs since the 1960s, nor in the cure of CD. However, with the advancement of metagenomics and metabolomics technologies, discoveries pertinent to various human conditions have been made, and for this reason, new light has begun to be shed on neglected tropical diseases [157]. Following this direction, in a review carried out by Kules et al. [158], the authors claim that high-throughput technologies, such as whole-genome sequencing, omics (metagenomics and metaproteomics), and mass spectrometry (MS), open new opportunities for the diagnosis of Vector-Borne Diseases. According to Kratz [159], even with all the recent advances associated with the discovery of drugs for CD, the likelihood of a new ideal treatment emerging in the coming years is still uncertain. The author claims that success in this discovery does not depend only on new tools and technologies, but also on the availability of financing and collaborative R&D models, in addition to a deepening of the understanding of the pathophysiology of the disease [159]. Financial support is needed for the initial stages of research and development of medication for CD [160]. In a work carried out by Calogeropoulou et al., the authors attest that many research programs are successful and have initial leads, however, they fall prematurely into the "valley of death" of candidate compounds. Therefore, they suggest that drug discovery for CD should become collaborative and include academic research, national and international organizations, government initiatives and private research centers as well as pharmaceutical companies [160].

Currently, modern drug design and discovery is based on computational methods that predict and evaluate binding of ligands to receptors related to various pathologies [161]. Therefore, the methods that are capable of promoting correct data acquisition, mining, and analysis are crucial in order to be able to obtain reliable results [162]. Traditional drug discovery and development is known to be time consuming and cost-intensive to both biotechnology and pharmaceutical companies [163]. According to Shaker et al. computer-aided drug design (CADD) offers methods to discover and optimize potent drugs in silico, aiming to screen millions of substances in order to identify chemical compounds that can geometrically and chemically bind to a specific cavity on a target protein [164].

In a study for drug repositioning, Sayé et al. used computational methods to discover substances with a structure similar to crystal violet (CV) [165]. CV was chosen because it has been used in blood banks for several years to eliminate the parasite *Trypanosoma cruzi*. The authors claim that the mechanism of action of CV is the inhibition of proline uptake by the parasite. Thus, the *in-silico* drug repurposing strategy through a similarity-based virtual screening protocol was able to identify compounds structurally related to CV (loratadine, cyproheptadine, olanzapine, and clofazimine). As a result, they observed that loratadine, cyproheptadine, and clofazimine inhibit the proline transporter TcAAAP069 and also they had a trypanocidal effect against all stages of *T. cruzi* [165].

2D QSAR is a tool that seeks to explain the relationships between chemical structures and experimental observations so that predictions of new compounds with certain desired properties can be made [166]. In a recent review, Halder and Dias Soeiro Cordeiro [168] analyzed different in silico approaches that were successfully applied in the discovery of anti-leishmaniasis (anti-LM) and anti-trypanosomiasis (anti-TP) drugs. After reviewing these works, the authors [168] identified two in silico approaches that have been little explored in recent years, but that had a high potential in the development of anti-LM and anti-TP agents, which are QSARs with.

In the search for molecules with action against *Trypanosoma cruzi*, [169] used the quantitative structure-activity relationship (QSAR) approach to investigate how molecular physical-chemical characteristics affect biological activity. These authors assumed that there are compounds that at some point show activity against *T. cruzi* but occasionally fail when tested in "whole-cell phenotypic assays." However, according to the authors, this result may be due to several factors such as inadequate physical-chemical and pharmacokinetic properties, resulting in molecules with little capacity to cross cell membranes. In this work, the authors applied artificial neural networks (ANNs) and kernel-based partial least squares regression (KPLS) to anti-*T. cruzi* activity data (Fig. 3). Through the analysis of atomic contribution maps, the authors found that fluorine and, in general, the heterocyclic aromatic rings and piperazine rings contributed positively to anti-T activity. Therefore, the integration of the ANN and KPLS analyses enabled the generation of a collection of key fragments strongly correlated with anti-*T. cruzi* compounds, providing valuable information to guide the design of new antichagasic agents with enhanced properties [169].

In 2012, Vincent et al. demonstrated the use of the metabolomic platform to elucidate the mode of action of an anti-trypanosomal drug [170]. According to Trochine et al., the first option for the treatment of CD is the administration of BZN, although its mode of action is not yet fully understood [171]. In order to analyze the metabolic response of *T. cruzi* to BZN, the authors [171] used a non-targeted MS-based metabolomics approach. Thus, the global changes in the metabolites that occur when BZN enters the parasite could be monitored. As a result, the authors concluded that treatment with BZN mainly affects molecules containing thiol in *T. cruzi*, therefore, this interference in thiol metabolism contributes to the action of the drug [171]. Later, the same group monitored, using the same metabolomic strategy, changes of low-mass metabolites in the epimastigote forms of *T. cruzi* treated with Bestatin [172]. Bestatin is a natural product with a broad-spectrum of inhibitory action on metalloaminopeptidases. Their results showed that Bestatin did not have a toxic effect directly on the parasite, but it had a substantial effect on the dipeptide pool, demonstrating its action as an inhibitor compound of dipeptidase enzymes [172]. In conclusion, the authors attested that the metabolomic platform has great potential for in situ analysis of enzymatic inhibition by pharmacological agents, and is an alternative for the evaluation of metabolic changes that occur after the exposure to a compound [172].

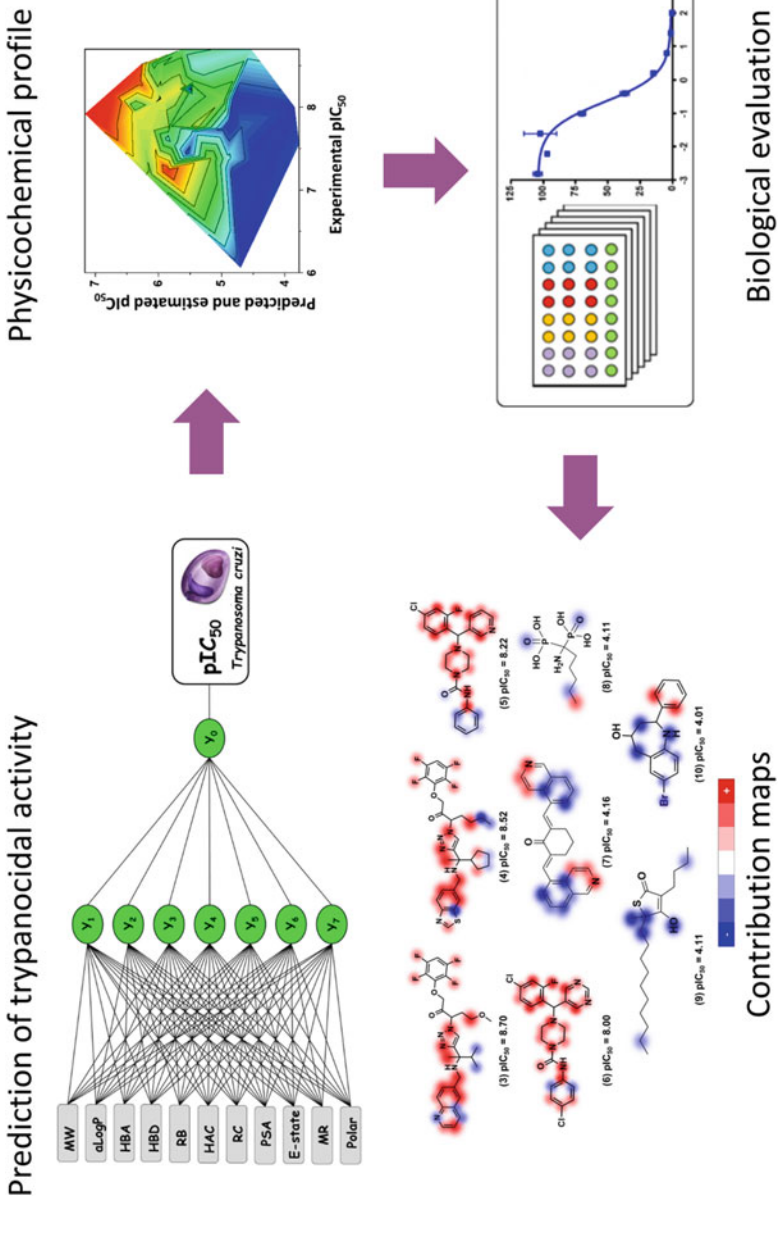



**Fig. 3** Proposed workflow for the design of new anti-*Trypanosoma cruzi* compounds based on the generation of contribution maps, prediction of trypanocidal activity using artificial neural networks and analysis of physical-chemical properties [169]

In a study on the metabolic changes that occur during the phases of exponential and stationary growth in *T. cruzi* epimastigotes, Barisón et al. studied the 47 metabolic intermediates of the most important pathways for energy metabolism and oxidative imbalance [173]. As a result, the authors showed for the first time that *T. cruzi* epimastigotes exhibit an adaptive metabolic mechanism that allows alternating the consumption of glucose to amino acids, in the transition from the exponential to the stationary phase. Mosquillo et al. carried out the first work that combined massive data from transcriptome and translatome, aiming to unravel the mechanism of action of two organometallic compounds (Pd-dppf-mpo and Pt-dppf-mpo) with trypanocidal activity [174]. As a result, the authors identified modified and/or metabolic enzymes present in the parasite, but absent in the mammalian host, which may become targets for rational drug planning. In a study conducted by Zrein and Chatelain on monitoring the status of CD in infected patients, the authors state that despite the great effort in basic research that seeks to help in the management of CD, to date there has been little translation into available products [175]. The authors pointed out that disease control becomes even more challenging due to the lack of methods that allow the effectiveness of treatments to be evaluated safely. Currently, patients infected with *T. cruzi* are diagnosed using antibody detection methods in serological assays [175]. However, the authors claim that it is essential to change the paradigm of serological methods in order to be able to monitor the elimination of the parasites correctly. Thus, they argued that analyzing the diversity of antibodies is more informative of the clinical status than the conventional serological tests that are designed for global detection of antibodies [175].

Therefore, to date, there are relatively few validated drug targets for CD. As seen in this section, the combination of different types of omics data (proteomics, genomics, transcriptomics, metabolomics, etc.) is necessary for a correct understanding of the functioning of the disease. As well as the resolution of how environmental factors and culture media can change the characteristics of the parasite under study. Thus, understanding the host–parasite relationship is fundamental for extracting useful information for the development of an effective drug against CD.

## 6 Perspectives: Challenges in New Drugs Discovery

Several recent reviews in the literature discuss this important point about the challenges present in new drugs discovery [16, 30, 56, 176–180].

According to the roadmap of Echeverría et al., prevention, diagnosis, and treatment are the three major levels of intervention for which it is necessary to seek solutions [1]. Progress has been made in the purpose of new drug discovery for treatment of CD. Parameters that need to be improved have been defined and joint actions bringing companies, support entities, and academies together have been established [181]. A successful drug discovery campaign typically takes 10–15 years [30, 56].

However challenges are still present such as a drug effective against the acute and chronic phase, difficulties in the translation process, genetic diversity of *T. cruzi*, development of standard tests to monitor the course of treatment with drugs to certify the cure of CD among other factors [16, 182]. New potential and promising targets, new methodologies including animal models of tropical disease infections that represent human disease are needed. Evolution in the standardization, as well as innovative strategies, is currently being developed to improve all process related to drug discovery [183]. Proteomic studies provide information on the pathogenic mechanisms of CD and other neglected tropical diseases, identifying molecular targets for drug discovery and development promising biomarkers to detect both stages of the disease and for potential use in diagnosis [184].

We can consider that despite all the difficulties, the panorama of discovering new drugs has improved in these years. A better understanding of the problems and challenges and greater integration between the pharmaceutical industries, academies, governmental and non-governmental intuitions are accelerating all the processes involved in developing new drugs to find the best treatment for Chagas disease and other neglected diseases.

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# Targeting Carbonic Anhydrases from *Trypanosoma cruzi* and *Leishmania* spp. as a Therapeutic Strategy to Obtain New Antiprotozoal Drugs


**Alessio Nocentini, Alane B. Vermelho, and Claudiu T. Supuran**

## Contents



**Abstract** Chagas disease and leishmaniasis are potentially life-threatening disorders, included in the list of neglected tropical diseases (NTD) by the World Health Organization. *Trypanosoma cruzi* and *Leishmania* spp. are protozoa of the Trypanosomatidae family, that are the etiological agents of the two parasitosis. The latter are also spreading significantly to Europe and North America, making urgent a concrete intervention from the healthcare systems of the developed countries. Carbonic anhydrases (CAs, EC 4.2.1.1) belonging to the α- and β-class were recently identified in these protozoans and shown to be essential in the pathogen physiology and pathogenicity with roles in growth, acclimatization, and virulence development. The α-CA from *T. cruzi* (TcCA) and the β-CA from *L. donovani chagasi* (LdccCA) have been recognized as new enzymatic targets for an

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antiinfective intervention overcoming the cross-resistance to existing drugs. This chapter gathered the state of the art on biochemistry and pharmacology of both protozoan CAs. All known inhibitors of TcCA and LdcCA are here illustrated and discussed in detail as for in vitro enzyme inhibition and in cell antiprotozoal action against multiple strains and developmental forms of *T. cruzi* and *Leishmania*.

**Keywords** Antiprotozoal, Carbonic anhydrase, Inhibition, NTD, Selectivity

## 1 Introduction

Chagas disease (American trypanosomiasis) and leishmaniasis are potentially life-threatening illnesses included in the list of neglected tropical diseases (NTDs) by the World Health Organization (WHO) (https://www.who.int/news-room/fact-sheets/detail/chagas-disease-(american-trypanosomiasis), https://www.who.int/health-topics/leishmaniasis#tab=tab_1) [1, 2]. These infections belong to the vector-borne diseases affecting 20 million people and killing more than 50,000 every year and are caused by parasites of the kinetoplastidae family (*Trypanosoma cruzi* and *Leishmania* spp) (https://www.who.int/news-room/fact-sheets/detail/chagas-disease-(american-trypanosomiasis), https://www.who.int/health-topics/leishmaniasis#tab=tab_1) [1, 2].

Kissing bugs of the Triatoma and Rhodnius genera naturally transmit *T. cruzi* that is primarily diffused in Latin America. Chagas disease progresses damaging organs in the cardiac, digestive, or neurological systems [1]. The bite of infected phlebotomines is instead the main cause of *Leishmania spp* transmission and potentially generates skin or visceral fatal damages. Leishmaniasis is the first-in-class NTDs in terms of mortality and morbidity [2].

The anti-protozoan agents available for the treatment of Chagas disease (the nitroheterocyclic compounds benznidazole and nifurtimox) and leishmaniasis (sodium stibogluconate, meglumine antimoniate, amphotericin B, paromomycin, pentamidine, miltefosine) exhibit high toxicity and limited efficacy, and resistance phenomena are constantly increasing worldwide [3, 4]. The poor interest shown by the pharmaceutical industry in searching new effective drugs for NTDs treatment is related to high costs and expected low financial return. On the contrary, a priority should exist in finding new approaches in the treatment of these parasitosis that started to spread more and more toward Europe and North America, urging a considerable attention from the healthcare systems of the developed countries (and from the drug companies) [5]. Large-scale analysis on the completely known genome sequence of both protozoans has recently provided the identification of new enzymatic targets, among which the carbonic anhydrases (CAs, EC 4.2.1.1) [6, 7].

$CO_2$ is a simple but crucial molecule in a *plethora* of physiological processes in organisms from all life kingdoms. Enzymes able to catalyze the $CO_2$ reversible

hydration – $CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$ – evolved throughout the tree of life to lead the spontaneously occurring reaction to meet cellular metabolic needs [8, 9]. These biological catalysts are the metalloenzymes known as carbonic anhydrases which are necessary to handle the high loads of the poorly water-soluble gas $CO_2$ produced in cells/tissues of most organisms. Also, CAs make the water-soluble reaction products $H^+$ and $HCO_3^-$ ions available to cells for pH regulation, ion homeostasis and metabolic processes, tightly controlled in all organisms/cells. CA isozymes have been found virtually in all mammalian tissues and cell types, where they actively intervene in $CO_2$ transport and numerous physiological processes [9, 10]. CAs are also present in plants, fungi, algae, protozoa, cyanobacteria, and bacteria [11–19]. Eight different, genetically distinct CA families are known to date, α-, β-, γ-, δ-, ζ-, η-, θ-, and ι-CAs [11–19]. Representatives of the enzyme classes α, β, γ, and ζ have been crystallized and structurally characterized in detail and show substantial three-dimensional variability between the different classes: overall shape of the molecules, protein folding patterns, and oligomeric organization. The catalytically active forms of α-, γ-, δ- and ι-CAs comprises a $Zn^{2+}$ ion tetracoordinated by three His residues and a water molecule/hydroxide ion, whereas in β- and ζ-CAs the amino acid ligands coordinating the metal ion consist of one His and two Cys residues. A Gln residue replaces one His ligand instead in η-CAs. Some of the catalytically active α-CAs also catalyze the hydrolysis of esters, and other hydrolytic reactions as well. No esterase activity was instead detected so far for enzymes belonging to other CA classes [11].

At present, infectious diseases are the second-leading cause of death in the world and the emergence of antiinfective-resistant microorganisms is an inevitable and widespread phenomenon inherent to most drugs [20–22]. CAs stood out in the scenario of research of antiinfective agents acting upon innovative mechanisms of action. In fact, it was shown that CAs are essential in the physiology and pathogenicity of several microorganisms with roles in growth, acclimatization, and virulence development. CA inhibition leads to growth impairment and defects which made CAs from pathogens suitable targets to fight infections [23–26].

The following paragraphs review biochemistry and pharmacology of the CA isoforms identified in *T. cruzi* and *L. donovani chagasi,* that are TcCA and LdcCA, respectively [6, 7]. TcCA and LdcCA were both cloned and characterized in 2013, and a chorus of inhibitors of these isoforms have been identified which represent potential antiprotozoal agents acting by a new mechanism of action that lack cross-resistance to existing drugs.

## 2 TcCA, the α-CA from *Trypanosoma cruzi*

*T. cruzi* encodes for an α-CA, identified, cloned, and characterized in 2013 [6]. The enzyme, named TcCA, was expressed using the Bac-to-Bac baculovirus expression system in *Spodoptera frugiperda* derived Sf9 insect cells. TcCA has a high catalytic activity for the $CO_2$ hydration reaction, being similar kinetically to the human

**Table 1** Kinetic parameters for the catalysis of the $CO_2$ hydration reaction for the human cytosolic isozymes hCA I and II, and isoforms TcCA and LdcCA measured at 20°C and pH 7.5 (for the α-CAs) and pH 8.4 (for the β-CA). The inhibition data with acetazolamide **AAZ** are also shown

| Enzyme | Species | Class | Catalytic activity | $k_{cat}$ ($s^{-1}$) | $k_{cat}/k_m$ ($M^{-1}$ x $s^{-1}$) | $K_I$ AAZ (nM) |
|---|---|---|---|---|---|---|
| hCA I | Human | α | Moderate | $2.0 \times 10^5$ | $5.0 \times 10^7$ | 250 |
| hCA II | Human | α | Very high | $1.4 \times 10^6$ | $1.5 \times 10^8$ | 12 |
| TcCA | *T. cruzi* | α | Very high | $1.2 \times 10^6$ | $1.5 \times 10^8$ | 61.6 |
| LdcCA | *L. donovani* | β | Medium | $9.3 \times 10^5$ | $5.9 \times 10^7$ | 91.7 |

isoform hCA II (Table 1, $k_{cat}/k_m$ of $15 \times 10^8$ $M^{-1}$ $s^{-1}$), although it is devoid of the His64 proton shuttle which is conserved in most α-CAs. His64 is replaced by an Asn residue in TcCA [11]. TcCA contains $Zn^{2+}$ ion in its active site, coordinated by three histidine residues and a water molecule/hydroxide ion [6]. In the next section all classes of inhibitors studied against TccA are presented and discussed in detail.

## 2.1 TcCA Inhibition

Sulfonamides are the main class of zinc-binding CA inhibitors [28–30]. Sulfonamides have been also the first antimicrobial drugs, discovered in 1935 by Domagk. These derivatives are used as diuretics for several clinical pathologies, such as glaucoma, epilepsy and as antimicrobial agents (https://www.who.int/health-topics/leishmaniasis#tab=tab_1). Sulfonamide/sulfamates CAIs are also used clinically as anti-obesity agents and are in advanced clinical trials for cancer treatment [30, 31] (https://clinicaltrials.gov/ct2/show/NCT03450018). A panel of 39 sulfonamides and one sulfamate (Fig. 1) were initially studied for the inhibitory characterization of TcCA (Table 2). Simple aromatic and heteroaromatic sulfonamides **1−24** were among them, as well as derivatives **AAZ−FAM**, which are clinically used drugs (https://www.who.int/health-topics/leishmaniasis#tab=tab_1) [6]. Acetazolamide (**AAZ**), methazolamide (**MZA**), ethoxzolamide (**ETZ**), and dichlorphenamide (**DCP**) are the classical, systemically acting CAIs. Dorzolamide (**DZA**) and brinzolamide (**BRZ**) are topically acting antiglaucoma agents. Benzolamide (**BZA**) is an orphan drug belonging to this class of pharmacological agents, whereas topiramate (**TPM**), zonisamide (**ZNS**), and sulthiame (**SLT**) are widely used antiepileptic drugs. Sulpiride (**SLP**), indisulam (**IND**), valdecoxib (**VLX**), celecoxib (**CLX**), saccharin (**SAC**), hydrochlorothiazide (**HCT**), and famotidine (**FAM**) were recently shown to belong to this class of pharmacological agents. Some such compounds showed promising inhibition constants ($K_I$s) in the range 61.6–93.6 nM. Of note, the subset of sulfonamides **1–10**, **15–18**, and **SAC** showed very weak TcCA inhibitory activity. Sulfonamides **11–14**, **19**, **21–24**, **DCP**, **ZNS**, and **HCT** were more effective with $K_I$ values in the range of 128–867 nM. The best sulfonamide TcCA inhibitors were the inhibitors: **20** ($K_I$ 88.5 nM), **AAZ** ($K_I$

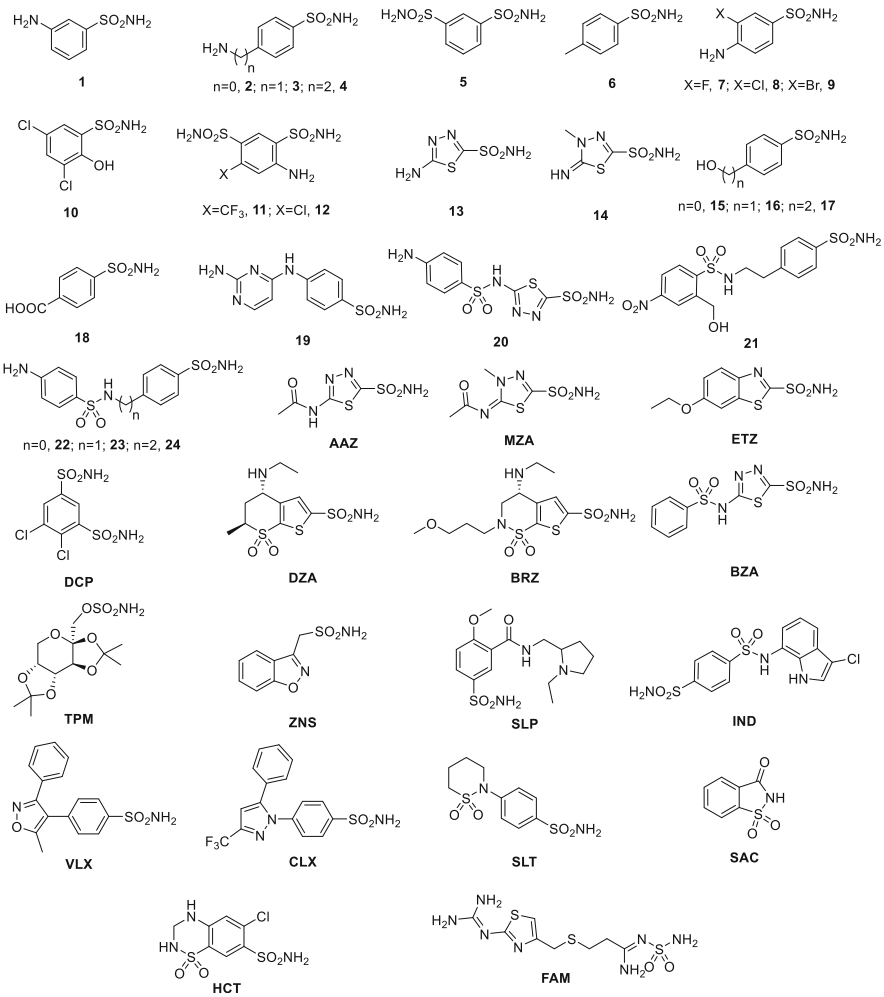



**Fig. 1** Sulfonamide/sulfamate CAIs **1–24** and **AAZ-FAM** investigated as TcCA and LdcCA inhibitors

61.6 nM), **EZA** ($K_I$ 88.2 nM), **DZA** ($K_I$ 92.9 nM), **TPM** ($K_I$ 85.5 nM), **SLP** ($K_I$ 88.5 nM), and **SLT** ($K_I$ 71.9 nM) (Fig. 2, Table 3).

A series of thiols was also evaluated as TcCA inhibitors, being well-documented that the mercapto moiety (in ionized, anionic form) may act as a good zinc-binding group (similar to the $SO_2NH^-$ one) for obtaining effective CAIs [29, 32]. Mercapto-derivatives **25–33** showed relevant TcCA inhibitory activity with $K_I$s in the range of 21.1–125 nM, and remarkable selectivity of action against TcCA over hCAs I and II. The best inhibitors were unsubstituted benzylidene **26** and the 3-methoxybenzylidene derivative **28** ($K_I$s of 21.1 and 34.5 nM). Compounds **26–33** were investigated in cell for their antitrypanosomal effects using

**Table 2** Inhibition profile of human isoforms hCAs I and II, and isozymes TcCA and LdcCA with sulfonamides **1–24** and clinically used sulfonamide/sulfamate CAIs **AAZ-FAM** by a Stopped-Flow assay [6, 27]

| Compound | $K_I$ (nM)[a] | | | |
|---|---|---|---|---|
| | hCA I | hCA II | TcCA | LdcCA |
| **1** | 45,000 | 295 | 25,460 | 5,960 |
| **2** | 25,000 | 240 | 57,300 | 9,251 |
| **3** | 25,000 | 170 | 7,231 | >100,000 |
| **4** | 21,000 | 160 | 9,238 | >100,000 |
| **5** | 28,000 | 300 | 63,800 | 8,910 |
| **6** | 78,500 | 320 | 44,200 | >100,000 |
| **7** | 8,300 | 60 | 8,130 | 15,600 |
| **8** | 9,800 | 110 | 6,925 | 9,058 |
| **9** | 6,500 | 40 | 8,520 | 8,420 |
| **10** | 7,300 | 70 | 9,433 | 9,135 |
| **11** | 5,800 | 63 | 842 | 9,083 |
| **12** | 8,400 | 75 | 820 | 4,819 |
| **13** | 8,600 | 60 | 534 | 584 |
| **14** | 9,300 | 19 | 652 | 433 |
| **15** | 6 | 2 | 73,880 | 927 |
| **16** | 164 | 46 | 71,850 | 389 |
| **17** | 185 | 50 | 66,750 | 227 |
| **18** | 109 | 33 | 84,000 | 5,906 |
| **19** | 95 | 30 | 810 | >100,000 |
| **20** | 690 | 12 | 88.5 | 95.1 |
| **21** | 55 | 80 | 134 | 50.2 |
| **22** | 21,000 | 125 | 365 | 136 |
| **23** | 23,000 | 133 | 243 | 87.1 |
| **24** | 24,000 | 125 | 192 | 73.4 |
| **AAZ** | 250 | 12 | 61.6 | 91.7 |
| **MZA** | 50 | 14 | 74.9 | 87.1 |
| **ETZ** | 25 | 8 | 88.2 | 51.5 |
| **DCP** | 1,200 | 38 | 128 | 189 |
| **DZA** | 50,000 | 9 | 92.9 | 806 |
| **BRZ** | 45,000 | 3 | 87.3 | 764 |
| **BZA** | 15 | 9 | 93.6 | 236 |
| **TPM** | 250 | 10 | 85.5 | >100,000 |
| **ZNS** | 56 | 35 | 867 | >100,000 |
| **SLP** | 1,200 | 40 | 87.9 | >100,000 |
| **IND** | 31 | 15 | 84.5 | 316 |
| **VLX** | 54,000 | 43 | 82.7 | 338 |
| **CLX** | 50,000 | 21 | 91.1 | 705 |
| **SLT** | 374 | 9 | 71.9 | 834 |
| **SAC** | 18,540 | 5,959 | 8,210 | >100,000 |
| **HCT** | 328 | 290 | 134 | 50.2 |
| **FAM** | 922.4 | 57.9 | 5,707 | nt |

nt: not tested

[a]Errors in the range of 5–10% of the reported data, from 3 different assays

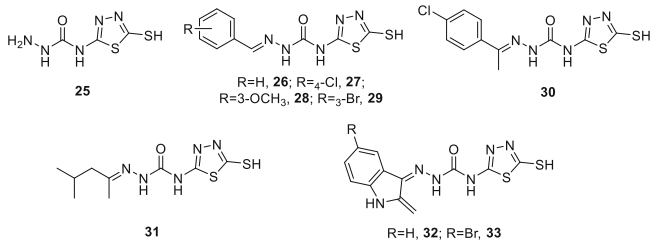



**Fig. 2** Thiols **25–33** investigated as TcCA and LdcCA inhibitors [6]

epimastigotes of *T. cruzi* strains DM28c and Y. All compounds (except **30**) inhibited the growth of both strains of *T. cruzi* at 256 μM concentrations, with a variable potency (inhibition of growth in the range 9–87% against strain DM28c and of 20–87% against strain Y). Benznidazole (**BNZ**) was, however, a stronger inhibitor of parasite growth compared to thiols **26–33**.

Always in 2013, Guzel-Akdemir et al. reported new aromatic/heterocyclic sulfonamides incorporating halogen/methoxyphenyl acetamide moieties (Fig. 3) which showed a potent, up to subnanomolar, TcCA inhibitory action with $K_I$s in the range of 0.5–12.5 nM (Table 4) [33]. As it occurred with hCA II, a thiadiazole ring bearing the sulfonamide moiety significantly enhanced the inhibition potency of derivatives **41–44**, that showed $K_I$s in the 0.51–0.95 nM range, with respect to benzensulfonamide compounds. However, these sulfonamides were ineffective as antitrypanosomal agents because of their highly polar nature and inability to cross biological membranes, in order to inhibit the parasite enzyme in cell. In 2015, Alafeefy et al. reported a new class of quinazoline-sulfonamides acting as efficient inhibitors against TcCA, with $K_I$s ranging in a low to high nanomolar range (Fig. 3) [34]. The best inhibitor of this series **46** showed a single-digit $K_I$ against TcCA ($K_I$ of 6.6 nM). However, the latter were not tested for their antitrypanosomal action in cell (Table 5).

Anti-protozoan agents, such as nitroimidazoles, exhibit a nitro aromatic group in their structure necessary for the drug activity. In 2018, Nocentini et al. studied a series of benzenesulfonamides including a nitro moiety on the aromatic scaffold (**51–70**, Fig. 4) against TcCA [35, 36]. The compounds showed sub- to low-micromolar $K_I$s (in the range 0.08–4.8 μM) and some selectivity for the target CA over hCAs I and II. A selected set of such derivatives was tested in cell against *T. cruzi* DM28c and Y strains but did not produce growth inhibition in the parasites (Table 6).

Driven by the evident low permeation issues of sulfonamides CAIs in *T. cruzi* cells, in 2018 Vermelho et al. explored the formulation of sulfonamide CAIs **35**, **36**, **40**, **42**, **43**, **43** as nanoemulsions (NEs) in clove (*Eugenia caryophyllus*) oil [37]. The approach was successful with several strong sulfonamide TcCA inhibitors finally showing significant antitrypanosomal effects against two different strains of the pathogen (Table 4). Relevantly, all compounds formulated as NEs showed a significantly greater efficacy than benznidazole against both *T. cruzi* strains. In contrast,

**Table 3** Inhibition profile of human isoforms hCAs I and II, and isozymes TcCA and LdcCA and antitrypanosomal/leishmanial data with thiols **25–33** [6]

| | $K_I$ (nM)[a] | | | | % Inhibition of growth | | | |
|---|---|---|---|---|---|---|---|---|
| Compound | hCA I | hCA II | TcCA | LdcCA | *T. cruzi* (DM28c) | *T. cruzi* (Y) | *L. chagasi* | *L. amazonensis* |
| **25** | 7,100 | 9,200 | 125 | 74.1 | nt | nt | nt | nt |
| **26** | 3,000 | >100,000 | 21.1 | 27.9 | 87 | 87 | 36.3 | 45.8 |
| **27** | 18,740 | 13,460 | 64.3 | 18.4 | 10 | 67 | 18.0 | 56.0 |
| **28** | 8,540 | 2,670 | 34.5 | 13.4 | 43 | 77 | 51.5 | 62.3 |
| **29** | 71,600 | >100,000 | 43.1 | 40.1 | 34 | 58 | 100 | 97.0 |
| **30** | >100,000 | 3,890 | 94.7 | 95.3 | ni | 32 | 32.2 | 76.4 |
| **31** | 8,530 | 8,850 | 52.4 | 19.5 | 20 | 43 | ni | 7.0 |
| **32** | 7,890 | 8,360 | 79.0 | 144 | 1,529 | 30 | 74.8 | 91.9 |
| **33** | 3,710 | 7,970 | 72.5 | >100,000 | 22 | 20 | ni | ni |
| **BNZ** | ni | ni | ni | nt | nt | 91 | ni | ni |

nt: not tested. ni: no inhibition. *T. cruzi* strains DM28c and Y epimastigotes were used for the in vivo experiments. Benznidazole (**BNZ**) was used as standard drug for the tests. Promastigote forms of *L. chagasi* MHOM/BR/1974/PP75 and *L. amazonensis* Raimundo strains MHOM/BR/76/Ma − 5 were used for the experiments. Concentrations of test compounds were in the range 2–256 μM

[a]Errors in the range of 5–10% of the reported data, from 3 different assays

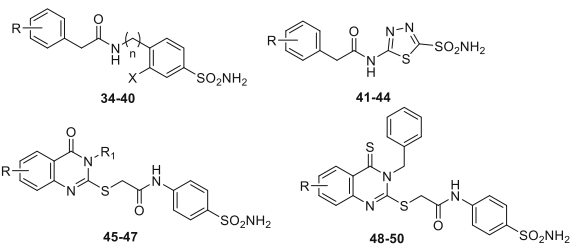



**Fig. 3** Sulfonamides derivatives **34–40** investigated as TcCA inhibitors [33, 34].

**Table 4** Inhibition profile of human isoforms hCAs I and II, and TcCA with sulfonamides **34–50** [33, 34]

| Compound | X | n | R | $R_1$ | $K_I$ (nM)[a] | | |
|---|---|---|---|---|---|---|---|
| | | | | | hCA I | hCA II | TcCA |
| **34** | H | 0 | 4-Cl | – | 346 | 104 | 2.7 |
| **35** | F | 0 | 4-Cl | – | 236 | 6.9 | 1.2 |
| **36** | F | 0 | 2-Br | – | 8.6 | 1.9 | 1.6 |
| **37** | Br | 0 | 2-Br | – | 6.5 | 0.80 | 10.9 |
| **38** | H | 1 | 2-Br | – | 4.3 | 0.77 | 9.0 |
| **39** | H | 2 | 4-F | – | 101 | 3.8 | 9.1 |
| **40** | H | 2 | 2-Br | – | 8.2 | 0.72 | 3.3 |
| **41** | – | – | 4-F | – | 223 | 3.2 | 7.6 |
| **42** | – | – | 4-cl | – | 4.9 | 0.70 | 0.95 |
| **43** | – | – | 2-Br | – | 7.6 | 0.70 | 0.83 |
| **44** | – | – | 4-$OCH_3$ | – | 2.9 | 0.76 | 0.51 |
| **45** | – | – | 5-$CH_3$ | Vinyl | 105 | 1.3 | 54 |
| **46** | – | – | 6-$CH_3$ | Benzyl | 86 | 1.7 | 6.6 |
| **47** | – | – | 8-$CH_3$ | Benzyl | 2078 | 208 | 35 |
| **48** | – | – | 5-$CH_3$ | – | 4,168 | 67 | 349 |
| **49** | – | – | 8-$CH_3$ | – | 2,162 | 114.2 | 666 |
| **50** | – | – | 8-$OCH_3$ | – | 803 | 65 | 56 |

[a]Errors in the range of 5–10% of the reported data, from 3 different assays

only compound **43** exhibited an $SI_{50}$ comparable to the reference drug and only against the Y strain. These effects are a probable result of the enhanced compound permeation to the protozoan cells by the NE formulation, that leads the CAIs to interfere with the life cycle of the pathogen, either by inhibiting pH regulation or carboxylating reactions in which $HCO_3^-/CO_2$ are involved. Flow cytometry showed that the sulfonamide NEs killed the parasites by necrosis, while apoptosis was triggered more efficiently than benznidazole only by compounds **36** and **44** against strain DM28c and compound **43** against strain Y.

Anion inhibitors and small molecules interacting with zinc proteins such as sulfamide, sulfamic acid, and phenylboronic/arsonic acids were also assayed against TcCA [38]. Several anions showed a low/medium micromolar inhibition range such as iodide, cyanate, thiocyanate, hydrogen sulfide, and trithiocarbonate ($K_I$s in the

**Table 5** Inhibition profile of human isoforms hCAs I and II, and isoforms TcCA and LdcCA with sulfonamides **51–70** [35, 36]

| Compound | R | $K_I$ (μM)[a] | | | |
|---|---|---|---|---|---|
| | | hCA I | hCA II | TcCA | LdCA |
| **51** | – | 0.91 | 0.24 | 0.08 | 0.21 |
| **52** | – | 4.35 | 0.18 | 0.16 | 0.34 |
| **53** | – | 4.79 | 0.84 | 2.5 | 4.7 |
| **54** | – | 6.21 | 0.64 | 0.11 | 0.39 |
| **55** | – | 6.18 | 0.61 | 0.24 | 0.46 |
| **56** | $C_6H_5$ | 1.38 | 0.39 | 3.5 | 3.9 |
| **57** | 4-F-$C_6H_4$ | 2.9 | 0.46 | 4.8 | 8.5 |
| **58** | $C_6H_5$ | >50 | 2.78 | 0.32 | 1.0 |
| **59** | 4-F-$C_6H_4$ | 5.39 | 0.53 | 0.46 | 0.98 |
| **60** | 4-$CF_3$-$C_6H_4$ | 5.2 | 0.2 | 0.51 | 2.3 |
| **61** | 4-F-3-$CH_3$-$C_6H_3$ | 7.58 | 0.21 | 0.38 | 3.0 |
| **62** | $C_6F_5$ | 0.69 | 0.27 | 0.28 | 1.4 |
| **63** | 3-$CH_3O$-$C_6H_4$ | 8.2 | 5.15 | 0.91 | 0.95 |
| **64** | 3,4-($OCH_2O$)-$C_6H_3$ | >50 | 4.3 | 1.02 | 2.0 |
| **65** | 3,5-$CH_3$-$C_6H_3$ | 8.33 | 0.45 | 0.69 | 1.9 |
| **66** | 3,5-$CF_3$-$C_6H_3$ | 5.99 | 1.72 | 1.35 | 3.6 |
| **67** | $CH_2CH_2C_6H_5$ | 9.29 | 3.08 | 0.74 | 0.86 |
| **68** | $CH_2$-(2-furyl) | >50 | 2.53 | 0.4 | 1.0 |
| **69** | 2,4,5-Triacetoxy-6-acetoxymethyl-tetrahydro-pyran-3-yl | 5.67 | 1.9 | 2.5 | 3.6 |
| **70** | 2,4,5-Trihydroxy-6-hydroxymethyl-tetrahydro-pyran-3-yl | 4.92 | 0.86 | 2.1 | 2.9 |

[a]Errors in the range of 5–10% of the reported data, from 3 different assays

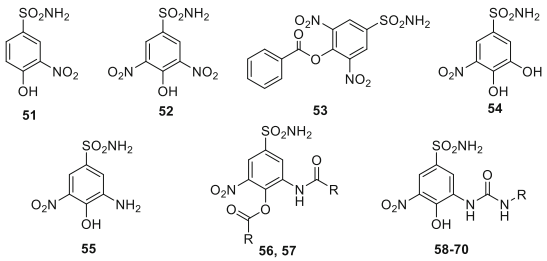



**Fig. 4** Sulfonamides derivatives **51–70** investigated as TcCA and LdcCA inhibitors [35, 36]

range of 44–93 μM), among which diethyldithiocarbamate stood out as the best TcCA inhibitor ($K_I$ of 5 μM).

In another study, poly(amidoamine) (PAMAM) dendrimers incorporating benzenesulfonamide moieties were investigated as inhibitors of a panel of CAs from pathogen microorganisms among which TcCA [39]. These multivalent derivatives exhibited low to high nanomolar inhibitory effects in the $K_I$ range 17.7–639 nM with a correlation with the PAMAM polymerization state.

**Table 6** $IC_{50}$ and $IC_{90}$ values derived from growth inhibition assays of epimastigotes of *T. cruzi* strains DM28c and Y, determination of cytotoxicity to RAW 267.4 cells ($CC_{50}$), and selectivity index ($SI_{50}$) of NEs of compounds **35**, **36**, **40**, **42**, **43**, **44** [37]

| | *T. cruzi* (DM28c) | | | *T. cruzi* (Y) | | | RAW 267.4 cells |
|---|---|---|---|---|---|---|---|
| Compound in NEs | $IC_{50}$ | $IC_{90}$ | $SI_{50}$ | $IC_{50}$ | $IC_{90}$ | $SI_{50}$ | $CC_{50}$ |
| **35** | 3.54 | 49.56 | 2.25 | 2.83 | >128 | 2.89 | 8.13 |
| **36** | 5.66 | 84.87 | 1.2 | 2.27 | 83.61 | 3.09 | 6.77 |
| **40** | 7.36 | 68.64 | 0.44 | 3.51 | 114.97 | 0.44 | 3.21 |
| **42** | 6.24 | 84.64 | 1.08 | 3.47 | 82.03 | 1.95 | 6.51 |
| **43** | 3.98 | 64.34 | 2.02 | 2.15 | 78.04 | 5.09 | 8.04 |
| **44** | 6.69 | 120.54 | 1.05 | 3.27 | 52.67 | 1.76 | 6.75 |
| **BNZ** | 20.63 | >128 | 5.54 | 21.92 | >128 | 5.89 | 125.74 |

$IC_{50}$: concentration (μM) which reduced the proliferation of epimastigotes by 50%. $IC_{90}$: concentration (μM) which reduced the proliferation of epimastigotes by 90%. $CC_{50}$: cytotoxic concentration (μg/ml) which reduced the proliferation of RAW 267.4 cells by 50%. $IS_{50}$: selectivity index $CC_{50}/IC_{50}$. Errors in the range of 5–20% of the reported data

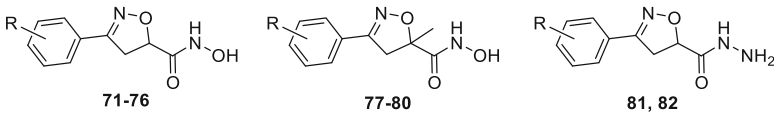



**Fig. 5** Hydroxamic acid derivatives **71–82** investigated as TcCA inhibitors [40]

Hydroxamic acid derivatives, that are hydroxamates, are able to coordinate the metal ion present in the CA active site [29]. Rodrigues et al. showed that hydroxamic acids could also act as inhibitors of the TcCA and of peptidases from this pathogen, such as metallo- and cysteine peptidase [40]. In detail, a series of 4,5-dihydroisoxazoles incorporating hydroxamate moieties (**71–82**, Fig. 5) were prepared and evaluated against TcCA (Table 7) and in cell/in vivo against several *T. cruzi* strains and forms. These assays recognized **76** as the lead compound with a $K_I$ of 39.8 nM (vs moderate inhibition of hCAs I and II) and complete growth inhibition of epimastigotes DM28c and Y at 32 μM concentration (Fig. 6). The results showed excellent values for inhibition of growth for all three developmental forms of the parasite at relatively low concentrations: $IC_{50}$ of 7.6 μM, 3.5 μM, <1 μM, respectively, against epimastigotes Y, trypomastigotes Y, amastigotes Y into THP-1 cells (Fig. 6). Compound **76** was not cytotoxic to macrophages (SI of 6.7). In particular, preliminary in vivo data using Balb/C mice infected with *T. cruzi* showed that **76** reduced bloodstream parasites and none of the mice treated with this compound died. A concentration of **76** that was one fourth of that standard of benznidazole drug was able to eliminate the parasitemia (Fig. 6). The authors also showed that **76** interferes with the activity of *T. cruzi* peptidases. A zymography assay showed that increasing concentrations of **76** decrease bands related to several metallopeptidases. The replacement of the hydroxamic acid moiety by an acyl hydrazine moiety (as in **81** and **82**) led to the complete loss of effects against TcCA and the parasite cells viability (Table 7 and Fig. 6), as well as their enzyme

**Table 7** Inhibition profile of human isoforms hCAs I and II, and TcCA with hydroxamic acid derivatives **71–82** [40]

| Compound | R | $K_I$ (nM)[a] | | |
|---|---|---|---|---|
| | | hCA I | hCA II | TcCA |
| **71** | 2-Cl | 516 | 27,900 | 263 |
| **72** | 3-Cl | 133 | 47,300 | 267 |
| **73** | 4-Cl | 3,240 | 94,500 | 189 |
| **74** | 2-OEt | 47,600 | 257 | 182 |
| **75** | 4-OEt | 64,000 | 3,810 | 141 |
| **76** | 4-OBn | 641 | 815 | 39.8 |
| **77** | 2-F | 598 | 733 | 615 |
| **78** | 3-F | 28,000 | 847 | 365 |
| **79** | 3-Cl | 72,200 | 297 | 94.1 |
| **80** | 3-Br | 47,000 | 808 | 71.3 |
| **81** | 2-OEt | >100,000 | >100,000 | >100,000 |
| **82** | 4-OEt | >100,000 | >100,000 | >100,000 |

[a]Errors in the range of 5–10% of the reported data, from 3 different assays

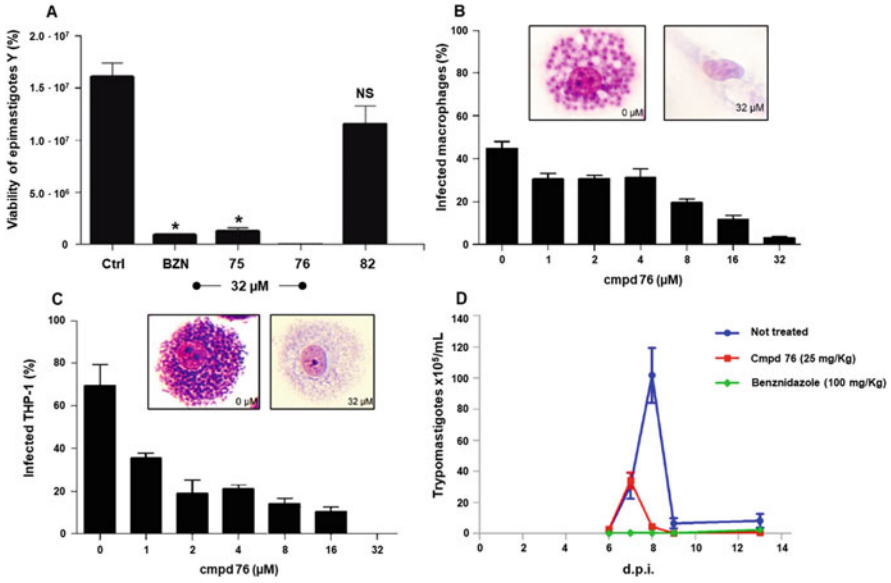



**Fig. 6** (**a**) Inhibition of growth of epimastigotes form (Y strain) of *T. cruzi* by benznidazole (**BZN**), **75**, **76**, **82** at concentration of 32 μM after 5 days of treatment. NS, nonsignificant vs control (CTL); BZN, benznidazole. Significant differences (*, $p < 0.05$) between untreated and treated cells using analysis of variance (ANOVA) by GraphPad Prism 5.0. Treatment with **76** reduces *T. cruzi* parasite burden in macrophages (**b**) and THP-1 cells (**c**). The data of the experiment was acquired by counting 300 cells per coverslip in duplicate that had been fixed and stained with Giemsa. (**d**) Balb/c mice infected with *T. cruzi* (Y strain) were treated with **76** for 7 days (comparison with **BZN**) (from 2 to 9 days postinfection, dpi)

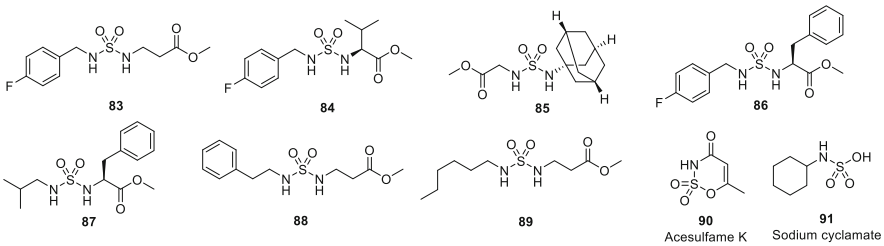



**Fig. 7** Ligands **71–82** identified in silico and investigated as TcCA inhibitors [41]

**Table 8** Inhibition profile of human isoforms hCAs I and II, and TcCA with derivatives **83–91**. Proliferation of *T. cruzi* Y strain epimastigotes and RA strain trypomastigotes treated with compounds **83–91** at 50 μM and 20 μM, respectively [41]

| | $K_I$ (nM)[a] | | Epimastigotes proliferation % | | |
|---|---|---|---|---|---|
| Compound | hCA II | TcCA | 3 days | 1 week | Trypomastigotes viability % 24 h |
| **83** | >10,000 | 594 | 86 | 92 | 73 |
| **84** | >10,000 | 604 | 79 | 100 | 66 |
| **85** | 4,957 | 752 | 100 | 94 | 91 |
| **86** | >10,000 | 950 | 57 | 55 | 72 |
| **87** | 8,528 | 261 | 73 | 96 | 100 |
| **88** | 8,884 | 7,250 | 100 | 100 | 76 |
| **89** | >10,000 | 449 | 72 | 100 | 98 |
| **90** | >10,000 | 2,242 | 92 | 89 | 100 |
| **91** | >10,000 | 348 | 89 | 78 | 52 |

[a]Errors in the range of 5–10% of the reported data, from 3 different assays

inhibitory action against TcCA and hCA I/II (Table 2, Fig. 5). These results indicate that hydroxamates of the **71–82** type act against both the parasite peptidases and CAs, which are essential enzymes for the parasite life cycle.

A structure-based in silico screening based on comparative modelling, molecular dynamics, and docking simulations allowed Llanos et al. to identify new TcCA inhibitors inducing weak hCA II inhibition, among which a set of sulfamides and the two sweeteners (**83–91**, Fig. 7) [41]. The derivatives showed medium nanomolar to low micromolar $K_I$s (261–7,250 nM) and a complete selectivity over hCA II (Tables 8 and 9). Some such compounds also showed a medium trypanocidal activity against *T. cruzi* epimastigotes and trypomastigotes (Table 8).

In two different studies from 2016 to 2019, Nocentini et al. reported a new CAI scaffold, that is the N-nitrosulfonamide, showing a potent and markedly selective action against isoforms from pathogens over ubiquitous hCAs [42, 43] (Fig. 8). N-Nitrosulfonamides have been designed according to the presence of the nitro group in the structure of many anti-protozoan agents, such as nitroimidazoles. A relevant efficacy, up to low-medium nanomolar range, was measured for derivatives **92–101** against TcCA ($K_I$s in the range 0.10–5.0 μM), while inhibiting hCAs

**Table 9** Inhibition profile of human isoforms hCAs I and II, and isozymes TcCA and LdcCA with N-nitro-sulfonamide derivatives **92–101** [42, 43]

| Compound | R | $K_I$ (μM)[a] | | | |
|---|---|---|---|---|---|
| | | CA I | CA II | TcCA | LdcCA |
| **92** | 2-$NH_2$ | 29.0 | 60.9 | 3.2 | 4.7 |
| **93** | 3-$NH_2$ | 54.7 | 7.7 | 0.15 | 0.49 |
| **94** | 4-$NH_2$ | 67.4 | 53.4 | 0.10 | 0.23 |
| **95** | 2-$N(CH_3)_2$ | 80.6 | 6.2 | 5.0 | 4.8 |
| **96** | 3-$N(CH_3)_2$ | 45.9 | 18.1 | 1.4 | 0.50 |
| **97** | 4-$N(CH_3)_2$ | 58.3 | 64.2 | 0.43 | 0.65 |
| **98** | $CH_2NH_2$ | 39.6 | 55.8 | 0.47 | 0.71 |
| **99** | 3-$NH_2$,4-OH,5-$NO_2$ | 19.8 | 45.0 | 0.85 | 1.0 |
| **100** | – | 7.3 | 2.9 | 0.35 | 0.52 |
| **101** | – | 4.9 | 2.2 | 0.32 | 0.44 |

[a]Errors in the range of 5–10% of the reported data, from 3 different assays

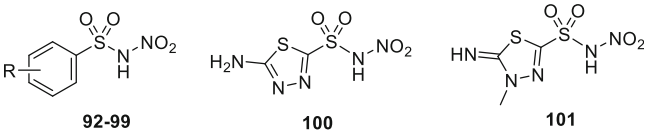



**Fig. 8** N-nitro-sulfonamide derivatives **92–101** investigated as TcCA and LdcCA inhibitors [42, 43].

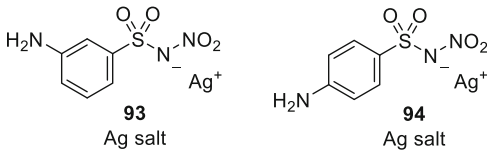



**Fig. 9** Ag salt form of derivatives **93** and **94** investigated in cell against various T. cruzi strains and developmental forms

I and II in a medium micromolar range ($K_I$s in the ranges 4.9–80.6 μM and 2.2–64.2 μM, respectively). Further, silver salts of all such derivatives were produced based on the marked effects against viruses, bacteria, fungi, and protozoa that silver salts have been shown to possess [44]. The biologically active silver ion ($Ag^+$) irreversibly damages key enzyme systems in the cell membranes of pathogens. Conversely, silver exhibits low toxicity in the human body, and minimal risk is expected due to clinical exposure. The silver salts $K_I$s measured against the same panel of CAs showed no in vitro modulation of the CA activity by the $Ag^+$ ion [43].

The silver salts of **93** and **94** (Fig. 9) showed to be more effective than benznidazole in inhibiting epimastigotes proliferation of both *T. cruzi* lineages DM28c and Y (Table 10). However, the two derivatives showed higher toxicity than **BNZ** against macrophages cells leading to SIs comparable to the reference

**Table 10** $IC_{50}$ values derived from growth inhibition assays of *T. cruzi* epimastigotes (strains DM28c and Y), trypomastigotes and intracellular amastigotes (strain DM28-luc); determination of cytotoxicity to RAW 267.4 and vero cells ($CC_{50}$), and selectivity index (SI) of compounds **93** and **94** as silver salts. Average values of three independent experiments. Errors in the range of 5–20% of the reported data

| | *T. cruzi* (DM28c) *epimastigotes* | | *T. cruzi* (Y) *epimastigotes* | | RAW 267.4 cells | *T. cruzi* trypomastigotes | | *T. cruzi* intracellular amastigotes | | Vero cells |
|---|---|---|---|---|---|---|---|---|---|---|
| Compound | $IC_{50}$ | SI[a] | $IC_{50}$ | SI[a] | $CC_{50}$ | $IC_{50}$ | SI[b] | $IC_{50}$ | SI[b] | $CC_{50}$ |
| **93** | 5.0 | 5.9 | 12.0 | 2.5 | 29.3 | 0.8 | 26.4 | 5.2 | 4.1 | 21.1 |
| **94** | 12.0 | 2.3 | 2.5 | 11.6 | 34.9 | 3.9 | 6.2 | 8.3 | 2.9 | 24.1 |
| **BNZ** | 29.1 | 4.8 | 17.0 | 8.1 | 137.5 | 15.6 | >32 | 1.7 | >294 | >500 |

[a]SI determined as CC50 against RAW 267.4 cells/IC50 against epimastigote forms
[b]SI determined as CC50 against Vero cells/IC50 against trypomastigote or amastigote forms

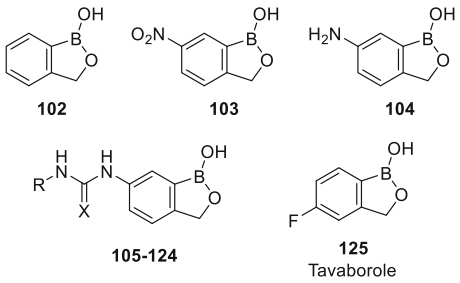



**Fig. 10** Benzoxaboroles **102–125** investigated as TcCA and LdcCA inhibitors [45–47]

drug. **93** and **94** also displayed 4- to 19-fold greater action than **BNZ** against *T. cruzi* forms relevant to human infection. Again, low SI values were calculated due to higher toxicity than **BNZ** to vero cells.

A relatively new inhibitory scaffold in the field of CAs was also investigated in 2018 for the inhibition of TcCA, that are the benzoxaboroles [45–47]. Only in 2016, the latter was shown kinetically and structurally to be able to inhibit CAs by a new mechanism of action. The benzoxaborole goes through a Lewis acid-base reaction with the zinc-bound hydroxide ion in the CA active site and the formed tetrameric ligand species -$B(OH)_2^-$ coordinates the metal ion [48]. A compound series including the simple benzoxaborole, a number of 6-substituted derivatives and tavaborole (Fig. 10), were investigated as TcCA inhibitors (Table 11). The α-class protozoan isozyme was inhibited by the benzoxaboroles only in a medium nanomolar range ($K_I$s in the range 16.6 to >100 μM).

## 2.2 TcCA Activation

In 2018, Angeli et al. carried out an activation study of the protozoan α-CA TcCA [49]. CA activators (CAAs) have been lately going through a second youth in drug discovery processes. Early evidence of the CA activation efficacy of amines, such as histamine, dating back to the 1940s was thereafter long debated up to deeming it an experimental artifact. In the early 1990s, the combination of highly purified enzymes and precise techniques such as the Stopped-Flow assay, put an end to the long controversy, testifying the undeniable existence of CAAs [10, 50]. To date, scientific evidence was gathered which testifies that CAs activation improves memory deficits, cognitive performance and learning, being nine of the fifteen human CA isoforms present in brain [51, 52]. Other evidence suggested that CAIs might impair memory in human, promoting the development therapeutic strategy based on CA activation for improving cognition, but also in therapeutic areas, such as phobias, obsessive-compulsive disorder, generalized anxiety, and post-traumatic stress disorders, for which few effective therapies are available. On the other hand, studying CA activation and identifying CAAs for isozymes from pathogens is important for

**Table 11** Inhibition profile of human isoforms hCAs I and II, and isozymes TcCA and LdcCA with benzoxaboroles **102–125** [45]

| Compound | X | R | $K_I$ (μM)[a] | | | |
|---|---|---|---|---|---|---|
| | | | hCA I | hCA II | TcCA | LdcCA |
| **102** | | | 5.69 | 8.18 | >100 | 3.79 |
| **103** | – | | 6.35 | 0.5 | >100 | 4.01 |
| **104** | – | | 9.43 | 0.6 | >100 | 2.37 |
| **105** | O | $CH_2Ph$ | 0.56 | 0.44 | 75.1 | 2.04 |
| **106** | O | $CH_2$-(3-Cl,5-$CH_3$-Ph) | 0.56 | 0.28 | 87.6 | 4.3 |
| **107** | O | Ph | 0.65 | 0.73 | 32.3 | 0.74 |
| **108** | O | 4-Cl-Ph | 3.46 | 0.71 | 37.8 | 0.62 |
| **109** | O | $CH_2$-fur-2-yl | 0.61 | 0.84 | 67.6 | 3.54 |
| **110** | O | 4-F-Ph | 0.23 | 0.48 | 24 | 0.78 |
| **111** | O | 4-$CF_3$-Ph | 0.49 | 0.46 | 60.7 | 2.59 |
| **112** | O | 2,4,6-Cl-Ph | 0.45 | 0.27 | 69.5 | 3.85 |
| **113** | O | 2-OMe,5-$CH_3$-Ph | 0.1 | 0.09 | 38.6 | 0.67 |
| **114** | O | 4-$COCH_3$-Ph | 0.29 | 0.8 | 33.6 | 0.48 |
| **115** | S | $CH_2CH_2Ph$ | 0.64 | 1.55 | 72.8 | 3.2 |
| **116** | S | 4-$CH_3$-Ph | 0.32 | 1.25 | 32.5 | 0.67 |
| **117** | S | 2-naphtyl | 0.55 | 1.15 | 58.8 | 3.06 |
| **118** | S | $OCH_3$-Ph | 0.51 | 1.25 | 12.6 | 0.59 |
| **119** | S | 4-$NO_2$-Ph | 0.38 | >100 | 46.8 | 0.91 |
| **120** | S | $CH_2Ph$ | 0.38 | 1.3 | 59.6 | 4.37 |
| **121** | S | 4-F-Ph | 0.35 | 1.5 | 19.7 | 0.66 |
| **122** | S | $CH_2$-fur-2-yl | 0.26 | 2.23 | 42.1 | 4.36 |
| **123** | S | 4-$CF_3$-Ph | 0.42 | 1.84 | 16.6 | 0.85 |
| **124** | S | Ph | 0.53 | 1.62 | 23.4 | 0.65 |
| **125** | – | | 2.01 | 0.46 | 60.5 | 2.54 |

[a]Errors in the range of 5–10% of the reported data, from 3 different assays

understanding the role that this enzyme has in the microorganism life cycle, particularly considering the fact that most activators identified to date (amine and amino acid derivatives) are autacoids present in rather high concentrations in different tissues of the host mammals that are infected by these parasites [49].

The best known CAA classes, the amino acids and aromatic/heterocyclic amines depicted in Fig. 11, were included in the activation study (Table 12). The best TcCA activators were L-/D-DOPA and 4-amino-L-phenylalanine **136**, which showed $K_A$s in the range 0.38–0.83 μM. Low micromolar activators were also L-/D-Trp, L-/D-Tyr, L-Gln, histamine, and serotonin ($K_A$s of 1.79–4.92 μM), whereas L-/D-His, L-/D-Phe, and L-Asp were less effective activators ($K_A$s of 6.39–18.7 μM). Amines such as dopamine, pyridyl-alkylamines **140–141**, aminoethyl-piperazine **142–143**, or L-adrenaline were devoid of activating effects on TcCA.

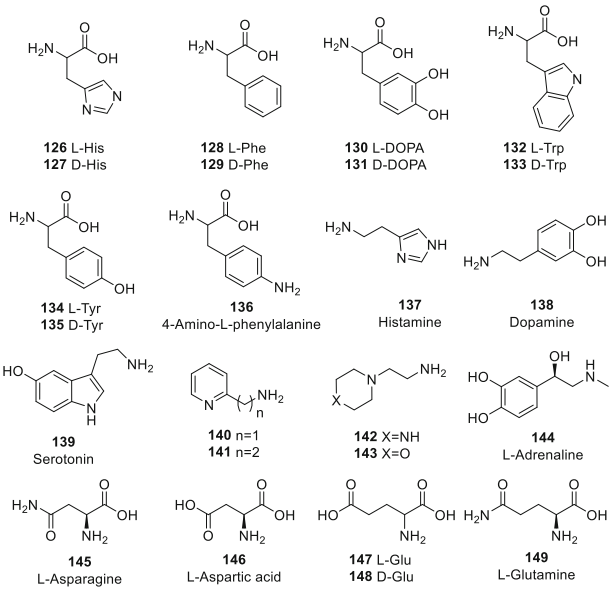



**Fig. 11** Amino acid and amine derivatives **126–149** investigated as TcCA and LdcCA activators

## 3 LdcCA, the β-CA from *Leishmania Donovani*

In *L. donovani chagasi* a CA, named LdcCA, was cloned and characterized. LdcCA is β-class CA with a medium range catalytic efficiency (Table 1, $k_{cat}$ of $9.35 \times 10^5$ $s^{-1}$ and $k_{cat}/k_m$ of $5.9 \times 10^7$ $M^{-1}$ $s^{-1}$ ) [7]. LdcCA was produced in the recombinant form using Sf9 insect cells which were transfected with the β-CA gene obtained from L. *donovani chagasi* cDNA. The expression was performed using the Bac-to-Bac baculovirus expression system. LdcCA contains $Zn^{2+}$ in its active site, coordinated by two Cys and one His residues. In 2015, Pal et al. analyzed the *Leishmania major* genome sequence (as well as the genomes of other species of Leishmania) predicting the presence of two putative CAs, LmCA1 and LmCA2 [53]. The authors also detected considerable CA activity in Leishmania cell lysates, thereby confirming the presence of functional CAs in *L. major*. One of these LmCAs is a β-CA, as that identified in *L. donovani chagasi* .

### *3.1 LdcCA Inhibition*

LdcCA was initially evaluated for the inhibition by the aromatic/heterocyclic sulfonamides (Fig. 1, Table 2) and 5-mercapto-1,3,4-thiadiazoles (Fig. 2, Table 3) shown earlier to act as effective inhibitors of TcCA [7]. Compounds **1–17**, **19**, and the clinically used **DCP**, **DZA**, **BRZ**, **BZA**, **TPM**, **ZNS**, **SLP**, **IND**, **VLX**, **CLX**,

**Table 12** Activation constants ($K_A$s) of hCA I, hCA II and the protozoan enzymes TcCA and LdcCA with amino acids and amines **126–149**

| Compound | $K_A$ (μM)[a] | | | |
|---|---|---|---|---|
| | hCA I | hCA II | TcCA | LdcCA |
| **126** | 0.03 | 10.9 | 11.3 | 8.2 |
| **127** | 0.09 | 43 | 7.5 | 4.1 |
| **128** | 0.07 | 0.013 | 12.1 | 9.2 |
| **129** | 86 | 0.035 | 6.4 | 3.9 |
| **130** | 3.1 | 11.4 | 0.8 | 1.6 |
| **131** | 4.9 | 7.8 | 0.4 | 5.5 |
| **132** | 44 | 27 | 2.5 | 4 |
| **133** | 41 | 12 | 1.8 | 6.2 |
| **134** | 0.02 | 0.011 | 4.9 | 8 |
| **135** | 0.04 | 0.013 | 2.8 | 1.3 |
| **136** | 0.24 | 0.15 | 0.7 | 15.9 |
| **137** | 2.1 | 125 | 2.7 | 0.7 |
| **138** | 13 | 9.2 | >100 | 0.8 |
| **139** | 45 | 50 | 2.0 | 0.6 |
| **140** | 26 | 34 | >100 | 0.2 |
| **141** | 13 | 15 | >100 | 0.012 |
| **142** | 7.4 | 2.3 | >100 | 0.009 |
| **143** | 0.14 | 0.19 | >100 | 0.9 |
| **144** | 0.09 | 96 | >100 | 4.9 |
| **145** | 11.3 | >100 | >100 | 4.8 |
| **146** | 5.2 | >100 | 18.7 | 0.3 |
| **147** | 6.4 | >100 | >100 | 12.9 |
| **148** | 10.7 | >100 | >100 | 0.08 |
| **149** | >100 | >50 | 2.8 | 2.51 |

[a]Errors in the range of 5–10% of the reported data, from 3 different assays

**SLT**, and **SAC** were not effective LdcCA inhibitors. Derivatives **18**, **20–24**, **AAZ**, **MZA**, **ETZ**, and **HCT** led instead to a significant Ldc inhibition with $K_I$ values in the range of 50.2–95.1 nM. Notably, the thiol derivatives 26–33 showed the most efficient inhibitory action against the β-class CA. In fact, of this series only compound **33** did not inhibit LdcCA. The lead semicarbazide derivative **25** was an effective LdcCA inhibitor ($K_I$ of 74.1 nM), but several such derivatives, that are **26–29** and **31**, showed even improved $K_I$s values in the range 13.4–40.1 nM. Again, a specific action of the thiols derivatives against LdcCA over hCAs I and II was relevantly detected. All compounds, except for the lead **25**, were tested in cell for the inhibition of promastigote forms of *L. chagasi* and *L. amazonensis*, at the concentration of 256 μM (Table 3). Inhibitor **29** was the most effective, inhibiting at 100 and 97% the growth of promastigotes, respectively. Transmission electron microscopy was performed with compound **29** at 256 μM to identify the antileishmanial effect of the inhibitor and ultrastructural changes that were observed in micrographs. Changes such as the appearance of electron-dense granules in the

**Table 13** $IC_{50}$ and $IC_{90}$ values (μM) derived from growth inhibition assays of epimastigotes of *L. amazonensis* and *L. infantum*, determination of cytotoxicity to RAW 267.4 cells ($CC_{50}$), and selectivity index ($SI_{50}$) of NEs of compounds **35**, **36**, **40**, **42**, **43**, **44**. Amphotericin B (**AMP**) was used as standard [54]

| | *L. amazonensis* | | | *L. infantum* | | | RAW 267.4 cells |
|---|---|---|---|---|---|---|---|
| Compound in NEs | $IC_{50}$ | $IC_{90}$ | $SI_{50}$ | $IC_{50}$ | $IC_{90}$ | $SI_{50}$ | $CC_{50}$ |
| **35** | 3.90 | 105.6 | 2.06 | 12.00 | n.d. | 0.66 | 8.13 |
| **36** | 12.01 | n.d. | 0.48 | 10.72 | n.d. | 0.77 | 6.77 |
| **40** | 10.55 | 92.74 | 0.34 | 12.46 | n.d. | 0.34 | 3.21 |
| **42** | 2.24 | 22.46 | 2.12 | 3.47 | 52.03 | 2.01 | 6.51 |
| **43** | 12.41 | n.d. | 0.64 | 14.58 | n.d. | 0.87 | 8.04 |
| **44** | 18.26 | n.d. | 0.37 | 51.70 | n.d. | 0.11 | 6.75 |
| **AMP** | 0.61 | 1.23 | 1.78 | 0.67 | 1.01 | 1.59 | 1.07 |

$IC_{50}$: concentration (μM) which reduced the proliferation of epimastigotes by 50%. $IC_{90}$: concentration (μM) which reduced the proliferation of epimastigotes by 90%. $CC_{50}$: cytotoxic concentration (μg/ml) which reduced the proliferation of RAW 267.4 cells by 50%. $IS_{50}$: selectivity index $CC_{50}/IC_{50}$. Errors in the range of 5–20% of the reported data

cytoplasm and in the flagellar pocket or the presence of many vesicles in the cytoplasm and the appearance of autophagic structures were observed.

Nocentini et al. studied the series of 3-$NO_2$-benzenesulfonamides **51–70** (Fig. 4) also against LdcCA [35]. The compounds showed promising sub- to low- micromolar $K_I$s (in the range 0.21–8.5 μM) and some selectivity for the target CA over hCAs I and II. A selected set of such derivatives was tested in cell against epimastigote forms of *L. amazonensis and L. infantum* strains but did not induce growth inhibition in the parasites.

The NEs in clove oil of sulfonamide CAIs **35–44** were also evaluated in cell against epimastigotes of *L. amazonensis* and *L. infantum* using amphotericin B (**AMP**) as standard drug (Table 13) [53]. Interesting inhibitory concentrations $IC_{50}$ were observed for some of the sulfonamides NEs, with values as low as 3.90 μM (**35**) and 2.24 μM (**42**) for *L. amazonensis* and 3.47 μM (**42**) for *L. infantum*. However, AMP was a stronger inhibitor of parasite growth compared to the NEs. Also some NEs displayed toxicity for macrophages (RAW 267.4 cells) higher than against the parasites, but lower than that induced by **AMP**. Hemolytic assay using human red blood cells also indicates that these NEs were less cytotoxic than **AMP**.

A zinc binder group of the sulfonamide type was also included as CAI moiety in two series of antileishmanial chalcogen-containing derivatives reported by Angeli et al., in research lines published in 2019 and 2020 [55, 56]. In a first study a wide set of organoselenium benzenesulfonamide derivatives, previously investigated for the inhibition of hCAs, were tested against LdcCA because of evidence gathered on the connection between selenium and trypanosomatids [55]. The most effective derivatives of the series (Fig. 12) showed a wide LdcCA inhibition range (Table 14) from low nanomolar to low micromolar $K_I$ values (0.006–7.8 μM).

All derivatives were evaluated in cell for their leishmanicidal activities against *L. infantum* amastigotes and for their cytotoxicities to human THP-1 cells

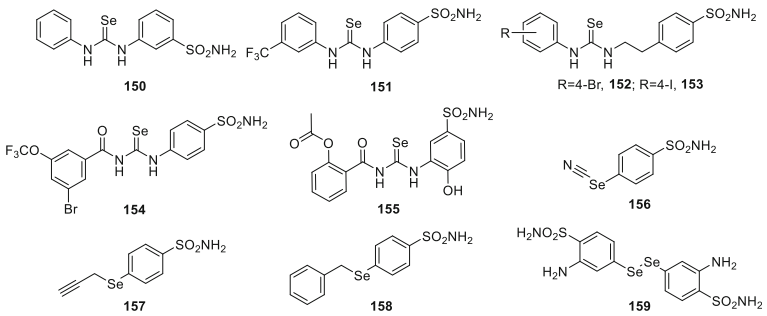



**Fig. 12** Benzenesulfonamide including selenium moieties **150–159** investigated as LdcCA inhibitors [55]

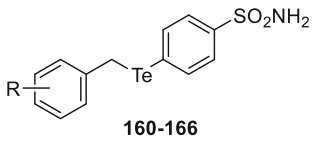
**Table 14** Inhibition profile of human isoforms hCAs I and II, and LdcCA and in cell antileishmanial data with sulfonamides **150–159** [55]

| | $K_I$ (nM)[a] | | | *L. infantum* amastigotes | THP-1 | |
|---|---|---|---|---|---|---|
| Compound | hCA I | hCA II | LdcCA | $IC_{50}$ | $CC_{50}$ | SI |
| **150** | 484 | 343 | 7.8 | 2.2 | 8.94 | 4.02 |
| **151** | 33 | 6.1 | 7.0 | 2.42 | 7.11 | 2.94 |
| **152** | 52 | 1.8 | 3.3 | 0.72 | 9.81 | 13.5 |
| **153** | 267 | 58 | 2.4 | 0.80 | >25 | 31.2 |
| **154** | 86 | 0.7 | 0.49 | 5.26 | 12.66 | 2.41 |
| **155** | 45 | 3.9 | 0.05 | 1.51 | 4.73 | 3.13 |
| **156** | 96 | 53 | 0.007 | 4.48 | >25 | >5.5 |
| **157** | 7.3 | 9.3 | 0.006 | 6.99 | >25 | >3.5 |
| **158** | 226 | 53 | 0.60 | 3.77 | >25 | >6.63 |
| **159** | nt | nt | 0.02 | 0.47 | 2.6 | 5.53 |
| **Miltefosine** | nt | nt | nt | 2.84 | 18.5 | 6.51 |
| **Edelfosine** | nt | nt | nt | 0.82 | 4.96 | 6.05 |

nt: not tested. $IC_{50}$: concentration (μM) which reduced the proliferation of amastigotes by 50%. $CC_{50}$: cytotoxic concentration (μg) which reduced the proliferation of THP-1 cells by 50%. SI: selectivity index $CC_{50}/IC_{50}$. Errors in the range of 5–20% of the reported data
[a]Errors in the range of 5–10% of the reported data, from 3 different assays

(Table 14). A subset of compounds, among which **152** and **153** showed submicromolar $IC_{50}$ values and greater SI (>8) than the reference drugs miltefosine and edelfosine.

In a second study, Angeli et al. included tellurium moieties in benzenesulfonamide derivatives as CAIs, to increase their antiprotozoal action on the basis of the antimicrobial and antiparasitic activity shown for tellurium against both flagellate and nonflagellate forms [56]. Compounds **160–166** (Fig. 13) were tested for the inhibition of hCAs I and II and a number of CA isozymes from human pathogen, among which LdcCA (Table 15). All telluride compounds were more potent and selective LdCA inhibitors with respect to **AAZ**, with $K_I$s of 9.1–24.7 nM. Derivatives **160–163**, **165,** and **166** were evaluated in cell against *L. Infantum*




**160-166**

**Fig. 13** Telluride sulfonamides **160–166** investigated as LdcCA inhibitors [56]

**Table 15** Inhibition profile of human isoforms hCAs I and II, and LdcCA and in cell antileishmanial data with sulfonamides **160–166** [56]

| Compound | R | $K_I$ (nM)[a] | | | $IC_{50}$ amastigotes | $CC_{50}$ THP-1 | SI |
|---|---|---|---|---|---|---|---|
| | | hCA I | hCA II | LdcCA | | | |
| **160** | H | 210 | 12.2 | 9.8 | 0.56 | 6.37 | 11.37 |
| **161** | 4-$CH_3$ | 24 | 4.4 | 9.6 | 3.44 | >25 | >7.26 |
| **162** | 4-$OCH_3$ | 1.5 | 2 | 31 | 0.73 | 1.98 | 2.71 |
| **163** | 2-naphthyl | 2401 | 182.3 | 24.7 | 1.47 | >25 | >17 |
| **164** | 4-$CH_3$ | 18 | 0.67 | 9.1 | nt | nt | nt |
| **165** | 2,6-di$CH_3$ | 256 | 3.3 | 9.4 | 2.23 | 6.64 | 2.98 |
| **166** | 3,4,5-tri$OCH_3$ | 377 | 13.2 | 9.6 | 0.02 | 6.01 | 300 |
| **Miltefosine** | – | nt | nt | nt | 0.82 | 4.96 | 6.05 |
| **Edelfosine** | – | nt | nt | nt | 2.84 | 18.50 | 6.51 |

nt: not tested. IC50: concentration (μM) which reduced the proliferation of amastigotes by 50%. CC50: cytotoxic concentration (μg) which reduced the proliferation of THP-1 cells by 50%. SI: selectivity index $CC_{50}/IC_{50}$. Errors in the range of 5–20% of the reported data
[a]Errors in the range of 5–10% of the reported data, from 3 different assays

amastigotes and for their cytotoxicity to human THP-1 cells (Table 15) using miltefosine and edelfosine as standard drugs. Among the compounds showing greater efficacy and selectivity than the references, derivative **166** markedly stood out both in potency ($IC_{50}$ of 0.02 μM) and specificity against the pathogen over human cells (SI of 300). Also, **166** showed the best antileishmanial activity against infected macrophages (Fig. 14a), did not affect the intestinal epithelium cells Caco-2 ($CC_{50}$ > 100 μM), while showing some toxic effects to Vero cells ($CC_{50}$ of 21.89 μM). The lead compound was also devoid of genotoxicity (as shown by the SOS/UMU test performed in *Salmonella typhimurium* bacteria) and exhibited a very low (2% approximately) oral bioavailability, which led the authors to adopt the intraperitoneal (i.p.) administration route for in vivo toxicity tests. Compound **166** was tolerated in mice at the dose of 45 mg/kg, while caused all animals death at a 90 mg/kg dose. Administration of **166** at 20 mg/kg *i.p.* for five consecutive days was tolerated in all tested animals. The anatomopathological inspections on liver specimens of the animal exposed to repeat dose toxicity study revealed normal morphology and no significant alterations associated with a cytotoxic effect (Fig. 14b). Kidney specimens reported a tubule nephrosis characterized by loss of nuclei of

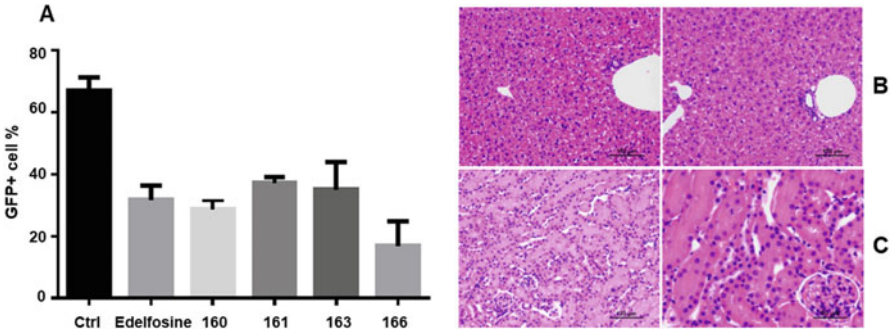



**Fig. 14** (**a**) Leishmanicidal activity on infected macrophages for compounds **160**, **161**, **163,** and **166** using edelfosine as standard drug. Histological samples of (**b**) liver and (**c**) kidney of mice treated with 20 mg/kg of compound **166** intraperitoneally for 5 days

**Table 16** $IC_{50}$ values derived from growth inhibition assays of epimastigotes of *L. amazonensis* and *L. infantum,* determination of cytotoxicity to RAW 267.4 ($CC_{50}$), and selectivity index (SI) of compounds **93** and **94** as silver salts. Average values of three independent experiments. Errors in the range of 5–20% of the reported data [43]

| | *L. amazonensis* | | *L. infantum* | | RAW 267.4 cells |
|---|---|---|---|---|---|
| Compound | $IC_{50}$ | SI[a] | $IC_{50}$ | SI[a] | $CC_{50}$ |
| **93** | 16.61 | 1.76 | 16.64 | 1.75 | 29.28 |
| **94** | 8.43 | 4.31 | 17.67 | 1.97 | 34.89 |
| **AMP** | 1.65 | 3.63 | 1.77 | 3.38 | 6.0 |

[a]SI determined as $CC_{50}$ against RAW 267.4 cells/IC50 against epimastigote forms

the cells that form the contiguous tubules, as well as the eosinophilia acquired by the cellular cytoplasm (Fig. 14c), which is in agreement with the cytotoxicity exhibited by **166** against Vero cells.

Nocentini and coworkers also investigated the set of N-nitrosulfonamides **92–101** depicted in Fig. 6 for the LdcCA inhibition [43]. As shown with TcCA, the derivatives reported high potency and selectivity for the protozoal CA over hCAs I and II (Table 9, $K_I$s in the range 0.23–4.8 μM vs 4.9–80.6 μM and 2.2–64.2 μM, respectively). The most active derivatives **93** and **94** as silver salts were assayed in cell for the growth inhibition of promastigotes of *L. amazonensis* and *L. infantum* using amphotericin B (**AMP**) as standard drug (Table 16). Both strains were inhibited by **93** and **94** in a low micromolar range, but less efficiently than **AMP**. However, SI values were comparable with respect to the standard because of the less toxic effect of the silver salts against RAW 264.7 macrophages.

The benzoxaboroles **102–125** (Fig. 9) evaluated by Nocentini et al. for the inhibition of the CA from *T. cruzi* were alongside assayed for the inhibition of LdcCA (Table 9) and showed a markedly greater efficacy as inhibitors of the β- over the α-CA, with $K_I$s mainly lying in a submicromolar range [45]. Indeed, the $K_I$s against LdcCA, from 0.48 to 4.37 μM, were comparable to those measured against hCAs and II, suggesting the benzoxaborole as a scaffold worth of further investigations to identify new antileishmanial agents.

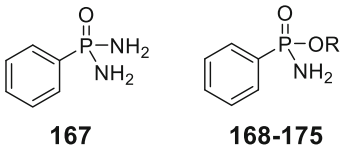



**Fig. 15** Benzenephosphonamidate **167–175** investigated as LdcCA inhibitors [57, 58]

**Table 17** Inhibition profile of human isoforms hCAs I and II, LdcCA with benzenephosphonamidate **167–175** [57]

| Compound | R | $K_I$ (μM)[a] | | |
|---|---|---|---|---|
| | | hCA I | hCA II | LdcCA |
| **167** | – | 77.8 | 32.8 | 2.1 |
| **168** | -$CH_3$ | 145 | 39.8 | 0.8 |
| **169** | -$CH_2CH_3$ | 339 | 160 | 3.5 |
| **170** | -$(CH_2)_2CH_3$ | 590 | 459 | 6.8 |
| **171** | -$CH(CH_3)_2$ | 730 | 349 | 5.1 |
| **172** | -$(CH_2)_3CH_3$ | 876 | 750 | 16.4 |
| **173** | -$(CH_2)_2OCH_3$ | 961 | 520 | 36.5 |
| **174** | -$(CH_2)_2Cl$ | 322 | 95.4 | 26.4 |
| **175** | -$CH_2CCH$ | 575 | 465 | 19.3 |

[a]Errors in the range of 5–10% of the reported data, from 3 different assays

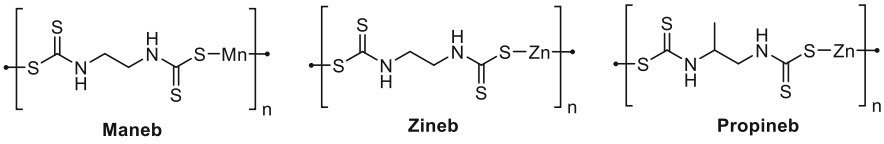



**Fig. 16** Metal dithiocarbamate complexes investigated as LdcCA inhibitors [53]

The same authors identified in 2020 another innovative chemical motif showing an inhibitory action against β-CAs among which LdcCA [57, 58]. In detail, phosphonamidates were recently validated as hCAs inhibitors and no phosphorus-based zinc-binding group had been assessed against β-class CAs. Phosphonamidates **167–175** (Fig. 15) showed low to submicromolar $K_I$s against (in the range 0.8–36.5 μM). hCAs I and II are instead only inhibited in a high micromolar range (32.8–961.2 μM), making phosphonamidates among the CAIs most selective for β-class over human isozymes known to date (Table 17).

Dithiocarbamates are another class of potent CAIs which also efficiently act against CAs from a number of pathogenic microorganisms. In 2015, Pal et al. firstly showed that dithiocarbamates can be chemotherapeutics against *Leishmania* parasites [53]. In fact, L. major promastigotes express functional CAs that can be inhibited by metal dithiocarbamates. Hence, three metal dithiocarbamate complexes that are **maneb**, **zineb**, and **propineb** (Fig. 16) were assayed against *L. major*

promastigotes and amastigotes showing a submicromolar dose-dependent inhibition of the parasite growth. Treatment with **maneb**, **zineb**, and **propineb** caused morphological deformities of the parasite and *Leishmania* cell death with LD50 values of 0.56, 0.61, and 0.27 μM, respectively [86].

### *3.2 LdcCA Activation*

The activation of LdcCA was explored using the panel of natural and nonnatural amino acids and amines **126–149** also used in the activation study of TcCA and depicted in Fig. 10 [59]. Also this study aimed at improving the understanding of the role of CA isoenzymes in the life cycle of protozoa such as *Leishmania* spp., being many of the investigated activators autacoids present in rather high concentrations in different tissues of the host mammals infected by these parasites. The most effective LdcCA activators belonged to the amine class, with histamine, dopamine, serotonin, 2-pyridyl-methylamine **140** and 4-(2-aminoethyl)-morpholine **143** showing $K_A$s in the range of 0.23–0.94 μM. 2-(2-Aminoethyl)pyridine **141** and 1-(aminoethyl)-piperazine **142** were even more effective activators ($K_A$s of 9–12 nM). Amino acids such as L-/D-His, L-/ D-Phe, L-/D-DOPA, L-/D-Trp and L-/D-Tyr were slightly less effective activators compared to the amines, but showed activation constants in the low micromolar range (1.27–9.16 μM).

## 4 Conclusions

Chagas disease and leishmaniasis are potentially life-threatening disorders included in the list of NTDs by the WHO. These parasitosis started to spread significantly in Europe and North America, urging a considerable attention from the healthcare systems of the developed countries. The CAs identified in the protozoans responsible for these diseases, that are TcCA (α-class CA) in *T. cruzi* and LdcCA (β-class CA) in *L. chagasi*, have been recognized as new enzymatic targets for an antiinfective intervention overcoming the cross-resistance to existing drugs. This chapter gathered the state of the art on biochemistry and pharmacology of both protozoan CAs. A *plethora* of inhibitors have been screened in vitro for TcCA and LdcCA inhibition and a significant subset of them were also tested in cell for the growth inhibition of multiple strains and developmental forms of *T. cruzi* and *Leishmania*, compared to the cytotoxicity exerted against human cell lines. Several markedly effective and selective CAIs have been identified to date which show really promising antitrypanosomal or antileishmanial actions and are worth of further investigations to set innovative therapeutic strategies to fight these NTDs.

**Compliance with Ethical Standards Conflict of Interest**: The authors declare that they have no conflict of interest.
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# New Compounds for the Management of *Trypanosoma brucei* Infection


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## Contents



**Abstract** The protozoan parasite *Trypanosoma brucei* causes human African trypanosomiasis (HAT), a fatal and neglected disease in the tropic areas. Owing to the scarcity of investments, new drug approvals, and resistance development to the current drugs, novel and selective targets are urgently needed to be explored. Trypanothione reductase, nitro-reductase, pteridine reductase 1, methionyl-tRNA synthetase, phosphodiesterases, and rhodesain represent emerging and attractive enzymes for the development of alternative anti-parasitic agents. The structure-activity relationships for each target were discussed as well as the correlation between in vitro and cell-based assays. These compounds could provide new therapeutic options for the limited arsenal of antitrypanosomal agents and are characterized by a good selectivity profile in terms of cytotoxicity against mammalian cells.

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## 1 Introduction

The denomination trypanosomiasis connotes a group of pervasive anthroponotic and zoonotic diseases transmitted by biting arthropods and caused by distinct species of flagellate protozoans belonging to the genus *Trypanosoma*, included in the Trypanosomatidae family, order Kinetoplastida of the subphylum Mastigophora.

Kinetoplastida are free-living or parasitic single-cell eukaryotic microorganisms, characterized by the presence of the kinetoplast, a mitochondria-like organelle containing a DNA structure termed kinetoplast DNA (kDNA), which is composed of two different types of DNA rings assembled in a unique chainmail arrangement [1]. They further share an ovoid to lanceolate body shape, usually with a helical symmetry, the nucleus, a microtubular membrane cytoskeleton, and at least one flagellum. In the Trypanosomatidae family, the main genera *Trypanosoma* and *Leishmania* encompass exclusively digenetic parasites, which may accomplish their life cycles within invertebrate and vertebrate hosts owing to adaptive differentiation forms. *Leishmania* and *Trypanosoma* parasites present high protein structural homology [2], similar genomic organization [3], common conserved cellular structure, and distinctive metabolic routes with respect to other eukaryotes [4], which may thus represent the boost for family-specific drug development. As for other alarming vector-borne diseases, human trypanosomiasis affects millions of people in the undeveloped tropical and sub-tropical areas of the world, causing acute illness, long-term sequelae, and early death in indigent communities recovering from colonialism. Furthermore, due to lack of economic return for pharmaceutical companies, funders, and politicians engaged in drug development, until very recently trypanosomiasis has remained largely under-researched, such that is still considered as a neglected tropical disease (NTD).

Based on the identity of the trypanosome and the transmitting vector species, two types of infections may be distinguished, characterized by different geographical distribution and clinical presentation, namely the South American trypanosomiasis and the African trypanosomiasis [5, 6]. Human African trypanosomiasis (HAT), commonly called sleeping sickness, affects humans and animals in several Countries of sub-Saharan Africa, where it ranks third behind malaria and filariasis [7, 8]. The disease is caused by two distinct but related subspecies of *Trypanosoma brucei* (subgenus Trypanozoon), transmitted via the bite of either the male or female blood-sucking Tsetse fly (*Glossina* spp.), and accordingly may occur in a dual clinical presentation: *T. brucei gambiense* is the parasite accounting for the chronic form of sleeping sickness, generally progressing slowly over an average time of 3 years, which is spread in western and central Africa and represents more than 98% of

global infections, whereas *T. brucei rhodesiense* is responsible for the fast-evolving syndrome, lasting from a few weeks to several months, which is endemic in eastern and southern Africa. However, a progressive geographical overlap of the two distinct varieties in north-western areas has been demonstrated. If not pharmacologically treated, both pathologies lead to the same clinical endpoint, consisting in mental deterioration, coma, and eventually death, resulting in high mortality rates, with a heavy impact on health and socio-economical systems of affected areas [9].

Although the genus *Trypanosoma* was previously recognized as a monophyletic group, the hypothesis of a paraphylogenetic relationship between American and African trypanosomes has been argued [10]. *T. cruzi* and *T. brucei* differ in their biology and pathogenesis, since the first is mostly intracellular, whereas *T. brucei* develops in extracellular districts of the host, such as blood and interstitial fluids. Moreover, the strictly related species *T. congolense* (subgenus Nannomonas), *T. vivax* (subgenus Dutonella), and *T. evansi* (subgenus Trypanozoon), and subspecies such as *T. brucei brucei* (subgenus Trypanozoon) can infect cattle, pigs, horses, camels, and wild ruminants, causing wasting animal trypanosomiases known as nagana, surra, dourine, and mal de caderas, which have a major impact on agricultural and farming economy of endemic regions.

Despite African trypanosomiasis was unrestrained after the 1960s, with an estimated overall 300,000 people infected, in recent decades coordinated control programmes and public health advances caused a 90% reduction in HAT incidence, with the number of new cases dropping below 10,000 in 2009, and up to less than 1,000 reports in 2018.

## 2 Clinical Relevance

Clinically, the disease presents two stages, that is the initial hemolymphatic phase, driven by trypanosome multiplication in the host lymph and blood districts, which evolves into the late meningoencephalitic stage, determined by the parasite invasion of the central nervous system [11, 12]. The earliest manifestation is a painful chancre at the bite site, much more frequently observed in travelers or tourists, followed by intermittent fevers, which reflect the parasite multiplication into the hematic district, headache, rash, lymphadenopathy and, most rarely, hepatosplenomegaly. Early diagnosis is difficult because of the lack of specific symptoms in this stage, apart the almost pathognomonic sign represented by the swelling of posterior cervical lymph node chain (the so-called Winterbottom's sign). The most common symptoms in the second stage include sleep disturbances, mainly consisting in the dysregulation of the sleep/wake cycle (hence the name "sleeping sickness"). In addition, various neurological and psychiatric disorders are manifested in this phase, as a consequence of growing involvement of central and peripheral nervous system (CNS, PNS) sites, such as apathy, ataxia, seizures, dyskinesia, irritability, and psychosis, and their progression over the time reflects the severity of the disease. Eventually, particularly in untreated patients, coma and death occur because of

major brain demyelination and atrophy. Similar to the CD, the heart may be affected, but only *T. brucei rhodesiense* is responsible for life-threatening pericarditis. Frequently, accurate diagnosis is feasible solely at this terminal stage, when therapy is limited to the few drugs enabled to cross blood-brain barrier (BBB).

## 3 The Life Cycle of *T. brucei*

The *T. brucei* cell cycle envisions several replicative and morphologically distinct stages of the parasite, which colonize specific tracts both in the invertebrate vector and the final mammalian host. The exogenous phase starts with the ingestion of infected blood, containing short stumpy (SS) trypomastigotes, by the hematophagous Tsetse fly [13]. In the insect midgut the parasites undergo cellular differentiation into procyclic (PC) trypomastigotes, followed by multiplication by binary fission; after leaving the midgut, PC trypomastigotes transform into epimastigotes (Es), which migrate to the fly salivary glands, where replication is continued before a further differentiation to a metacyclic (MT) trypomastigote form occurs. The host-cycle is initiated when parasites are inoculated by the fly into the human skin during the subsequent blood meal. The MT trypomastigotes enter the human hemolymphatic system, where they differentiate into a long slender (LS) form; LS trypomastigotes then travel throughout the body and may accumulate in adipose tissue and skin. They eventually reach the spinal fluid, giving rise to the CNS invasion which is responsible for the severe neurological and psychiatric complications seen in the late stage of HAT. If the parasite load in the bloodstream increases, LS trypomastigotes further differentiate into the SS non-replicative form, which is adapted for transmission and survival inside the insect vector [14].

*T. brucei* persistence within the host is strictly dependent on its metabolic adaptability: it was observed that whilst PC stages are preferably dependent on fatty acid metabolism, the blood forms rely on glycolysis for ATP production and anabolic pathways [15]. Major advances in host-parasite metabolomics will pave the way for the identification of enzymatic pathways sensitive to inhibition, thus leading to novel drugs development and clinical implementation. Trypanosomes share elongated cell shapes, defined by similar cytoskeletal architectures [16, 17]. Most endo- and exocytotic events in the cell are governed by the flagellar pocket [18], which is devoid of the rigid microtubule array: this critical component, made up by a membrane invagination at the flagellum posterior end, plays a key role in nutrient acquisition, protein secretion, removal of surface-bound immune effectors (as the variant surface glycoprotein, VSG) [19], and drug sensitivity [20], beyond being involved in cell division and morphogenesis. Thus, exploitation of drug internalization approaches, based on interference with the endocytic trafficking, represents an attractive opportunity [21, 22].

## 4 Current Status in Antitrypanosomal Therapy

Past decades have seen remarkable successes in the management of human trypanosomiasis [23, 24]. However, available drugs suffer from several drawbacks, with only few agents approved, largely represented by old compounds that display adequate efficacy albeit confined to only a single clinical stage. Even more, they often present unfavorable toxicity profiles and emergence of resistance, and mostly require parenteral administration, with complex regimens to apply in rudimentary health systems.

The standard protocol for the hemolymphatic stage in the *T. brucei gambiense* infection consists in pentamidine by parenteral route; suramin is recommended only for the disease caused by *T. brucei rhodesiense* (Fig. 1), due to the risk of severe adverse reactions upon suramin treatment in patients of *T. brucei gambiense*-endemic areas, which are frequently co-infected with onchocerciasis [25]. Unfortunately, owing to their highly hydrophilic and polar character, both drugs are trapped outside the BBB and are no longer effective once the parasites have invaded the CNS.

Pentamidine (Fig. 1) is the representative drug of the aromatic diamidine class; this water-soluble di-cationic molecule can enter the parasite only by means of membrane transporters, among which the adenine nucleobase/nucleoside (P2) transporter, the high-affinity pentamidine transporter (HAPT1) and the low-affinity pentamidine transporter (LAPT1), all expressed in bloodstream forms but not in PC trypomastigotes [26]. Development of pentamidine resistance and

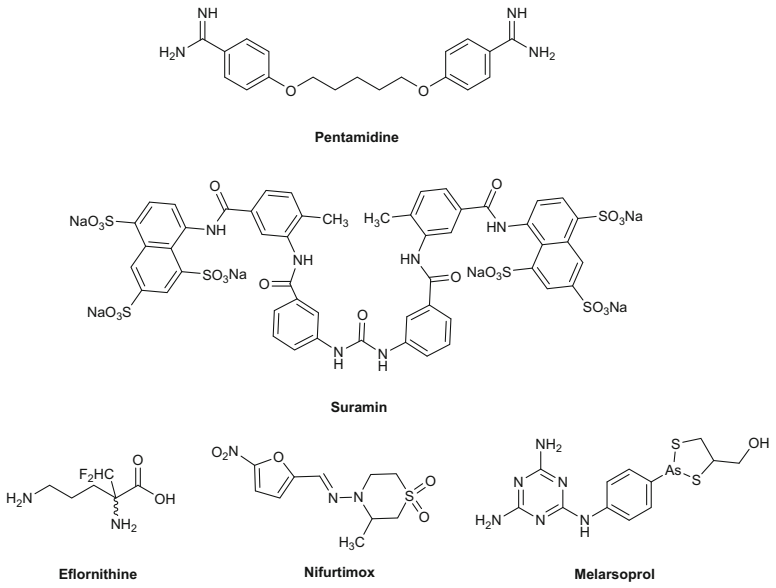


**Fig. 1** Structures of the current arsenal in clinical use

cross-resistance with other antitrypanocidals is deeply affected by expression levels of these proteins. HAPT1 was later hypothesized to correspond to aquaglyceroporin 2 (AQP2) and found to be the main determinant of melarsoprol-pentamidine cross-resistance (MPXR) [27]. This porine channel, localized in the flagellar pocket of bloodstream trypanosomes, has been recently suggested also as a pentamidine target [28].

Several mechanisms for the trypanocidal action of the drug have been proposed: like other diamidines, pentamidine binds to the DNA double helix of the *Trypanosoma*, at the level of adenine-thymine-rich regions in the minor groove, forming cross-linkages between two adenines four to five base pairs apart. Again, by acting as a reversible inhibitor of trypanosomal *S*-adenosylmethionine decarboxylase (SAMDC), it was shown to interfere with the synthesis of polyamines [29]. Possible other modes of action include kinetoplast fragmentation, and inhibition of the biosynthetic pathways for proteins and phospholipids [30]. Furthermore, the drug ability to inhibit mitochondrial topoisomerases, which results in a damage of the organelle genome, has been found to be operating for trypanosome as well as for *Pneumocystis jirovecii* mitochondria; that is the reason why pentamidine, in the current isethionate form, was marketed in the mid-1980s as effective medication for the opportunistic infections commonly affecting patients with AIDS. Despite non-negligible, undesirable reactions, such as abdominal pain and hypoglycemia, pentamidine is in general well tolerated.

Conversely, suramin is known to provoke significant side effects, among others hypersensitivity, agranulocytosis, and nephrotoxicity. This over 100-year-old drug is a symmetric polysulfonated naphthylurea compound (Fig. 1), which is completely dissociated at physiological pH and as such unable to cross the membrane phospholipidic bilayer by passive diffusion. Some authors proposed that low-density lipoproteins (LDLs), abundant in the membrane at the flagellum and flagellar pocket of trypanosomes, may act as low- and high-affinity transporters of suramin [31]. Although controversial, this study yet supports the shared idea that suramin is uptaken into the parasite cell by a drug-specific, receptor-mediated endocytotic event. The trypanocidal activity of suramin partly results from the selective inhibition of *T. brucei* glycolytic enzymes and consequent disruption in ATP generation [32]. Selectivity is driven by the electrostatic interactions formed between the large, polyanionic suramin molecule and the unique, paired clusters of basic amino acids at enzyme surfaces [33].

At present, the preferred medications for the second-stage of trypanosomiasis are represented by eflornithine and the more toxic melarsoprol. Eflornithine is only effective against *T. brucei gambiense*, and is generally used with nifurtimox in combination therapy, which appears to be more valid and safer in comparison with eflornithine monotherapy. Initially developed as anticancer drug, eflornithine was repurposed by Bacchi and co-workers as HAT late-stage treatment, owing to its ability to cross the BBB [34]. The drug, corresponding to racemic α-difluoromethylornithine (DFMO) (Fig. 1), presents structural analogy with the amino acid ornithine, and can exploit the trypanosome amino acid transporter AAT6 to be introduced into the cell [35]. Ornithine enters into the parasite polyamine biosynthetic

pathway as ornithine decarboxylase (ODC) substrate and crucial precursor of spermidine, which is incorporated into trypanothione, i.e. the protozoan correlative of mammalian glutathione. As mechanism-based inhibitor of ODC, eflornithine blocks the biosynthesis of polyamines crucial for the parasite [36]; the consequent cellular accumulation of ornithine, *S*-adenosylmethionine (SAM), and decarboxylated *S*-adenosylmethionine (dcSAM) finally leads to interference in the methylation reactions of proteins, nucleic acids, and lipids [37, 38]. DFMO shows no trypanocidal activity on *T. cruzi*, which lacks ODC being auxotrophic for short chain polyamines. Human-to-protozoan selectivity relies on the different turn-over rates of corresponding ODCs. Very interestingly, eflornithine has been shown to prevent hirsutism in women, and several pharmaceutical companies have considered the opportunity to exploit this secondary effect to market the drug for this new therapeutic use [39].

Nifurtimox (Fig. 1) is the drug of choice for the oral treatment of acute forms of the South American trypanosomiasis. On account of the presence of the nitro group, similarly to other nitrofurans the drug undergoes metabolic reactions of both reductive and oxidative type, leading to the generation of a variety of reactive radicals (superoxide, hydroperoxide, hydroxyl) prone to react with parasite macromolecules, thus ultimately killing the pathogen [40]. The identification of the mitochondrial type 1 NADPH-dependent nitro-reductase (NTR1) of the parasite, and the evidence that the enzyme activity is essential for nifurtimox to be effective in *T. brucei* and *T. cruzi* infections, substantiated the drug mechanism of action. An alternative pathway was proposed, which envisions the opening of the furan ring in the hydroxylamine intermediate and consequent exposure of an open-chain nitrile, which represents a highly cytotoxic group [41].

Melarsoprol recommendation is currently limited to first-line treatment for the *rhodesiense* form, since the *gambiense* infection may be more efficaciously managed with eflornithine. Melarsoprol (Fig. 1) is an organic trivalent arsenical prodrug made of melaminophenyl-arsine complexed with the metal chelator moiety of dimercaptopropanol (British anti-Lewisite or BAL, dimercaprol), to diminish the metal-associated toxicity. The active metabolite melarsen oxide, once internalized by the P2 adenosine/adenine transporter, as many trivalent arsenical compounds which display high affinity for vicinal sulfhydryl groups, rapidly reacts with the dithiol form of trypanothione, an oxidative and chemical stressors scavenger unique to kinetoplastid flagellates [42]. The stable adduct represents a competitive inhibitor of trypanothione reductase (TR), which parallels human glutathione reductase in the regulation of the thiol/disulfide pool in the parasite. The synthetic pathway of trypanothione, containing the polyamine spermidine, is interfered by ODC inhibitors, so that eflornithine and melarsoprol have been proposed as acting in synergism. Other plausible mechanisms of action include inhibition of phosphogluconate dehydrogenase and key glycolytic enzymes, such as pyruvate kinase [43]. Melarsoprol presents many adverse effects, the most severe of which is a post-treatment reactive encephalopathy (PTRE), occurring in approximately 10% of the patients, with a case-fatality rate of up to 50%. Unfortunately, parasite resistance to melarsoprol, due to mutations affecting AQP2, emerged as early as the 1970s, and is now widespread.

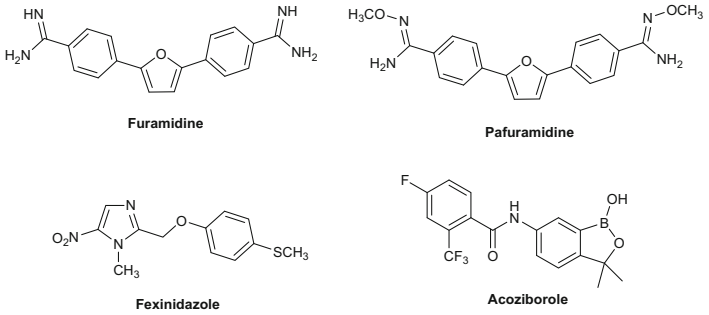


**Fig. 2** New emerging drugs in clinical trials

Aryl diamidine analogs of pentamidine, namely furamidine and its orally available prodrugs (pafuramidine) (Fig. 2), have been developed. Similar to the prototype, they bind to AT-rich sequences in kDNA [44]. Pafuramidine maleate, designed as the methoxy prodrug of furamidine, was a promising candidate for the oral treatment of the hemolymphatic stage, but unfortunately failed due to the insurgence of liver and renal toxicity in the course of retrospective phase I safety trials [45].

A significant improvement in the management of HAT caused by *T. brucei gambiense* has been the development of the nifurtimox-eflornithine combination therapy (NECT) and other combination treatments. However, NECT protocols require hospitalization and trained nursing staff to be effected. In 2018 the innovative molecule fexinidazole (Fig. 2) received a positive scientific opinion by the European Medicines Agency (EMA) and was added to the World Health Organization's List of Essential Medicines in 2019 [46–48]; this oral drug, developed under the coordination of the DNDi (Drugs for Neglected Diseases Initiative) and recommended as first-line for gambiense sleeping sickness, has recently been submitted to clinical trials to further assess its efficacy and safety for rhodesiense sleeping sickness.

As a nitro-imidazole derivative, fexinidazole represents a bioprecursor undergoing activation by the NADH nitro-reductase of the parasite (*Tb*NTR1), which leads to generation of radicals and oxidative stress. In the body, fexinidazole is converted to sulfoxide and then sulfone derivatives, both active against *T. brucei* [49].

Another promising candidate, expected to be administered in a single oral dosing for the treatment of both disease stages, is acoziborole (Fig. 2), a novel molecule which is currently undergoing Phase III trials. This compound, belonging to the class of 6-carboxamido-benzoxaboroles, exerts its trypanocidal activity through inhibition of mRNA maturation, by targeting the cleavage and polyadenylation specificity factor 3 (CPSF3) [50]. SAR extensive studies on 6-substituted-benzoxaboroles have established the key requirement of the boron atom inserted into the heterocyclic scaffold for trypanocidal activity [51].

## 5 New Therapeutic Strategies and Emerging Targets

In the recent period, the involvement of pharmaceutical industries in NTDs is expanding, and anti-trypanosomial drug discovery can benefit from joint ventures between companies, academias as well as governmental and non-profit organizations. Even so, there are very few candidates in clinical development, despite the availability of safer and oral treatments, with an activity range extended to both clinical stages, would be transformative for HAT eradication in socioeconomically vulnerable populations. Currently, there are no human vaccines available for this parasitosis [52].

Phenotypic compound screenings and drug repurposing strategies have prevailed in discovering new trypanocidal molecules compared to target-based approaches [53]. Success in phenotypic approaches is best exemplified by the two promising candidates fexinidazole and acoziborole, which are currently undergoing clinical trials. Drug repositioning offers a number of advantages in NTD reduction strategies, since the use of approved agents to new clinical application may capitalize on available information regarding clinical safety, pharmacokinetics, and pharmacodynamics, leading to time and cost saving. For instance, nifurtimox, developed at first for the oral treatment of severe CD, has been repurposed as a remedy in the HAT second-stage in combination therapy with eflornithine (NECT), to limit partner drug toxicity. Nevertheless, at present target-based drug discovery may benefit from the completion of *T. cruzi* and the *T. brucei* genome sequences, as well as from advances in bioluminescence-based in vivo assays and CRISPR/Cas9 technology.

From an evolutionary point of view, trypanosomatids lineages diverged very early from the main eukaryotic phylogenetic lines, as confirmed by the unique molecular processes characterizing these parasites that can be potentially druggable [54]. This evidence has prompted the exploitation of several trypanosomatid-specific enzymatic pathways as potential targets, including glycolysis, folate metabolism, redox balance, and purine salvage. Furthermore, parasite peptidases have emerged as crucial proteins to be targeted in the search of new drugs, as exemplified by cathepsin-like enzymes, and serine proteases such as oligopeptidase B and prolyl oligopeptidase [55]. Also, endocytosis and transmembrane proteins involved in drug transport may represent valuable targets.

Uniquely, in trypanosomatids most glycolytic enzymes are compartmentalized into the glycosome, an organelle belonging to the peroxisome family. Glycosomes have been shown to be essential for ATP production and growth of the bloodstream forms of African trypanosomes, hence the targeting of enzymes such as phosphofructokinase is a promising approach. New perspectives for the treatment of sleeping sickness envision the feasible alteration of the *T. brucei* cell-surface glycans, consequent to the inhibition of glycosyltransferases and glycosidases [56, 57]. Furthermore, at least in *T. brucei*, two enzymes involved in the parasite folate cascade, namely dihydrofolate reductase (DHFR) and pteridine reductase 1 (PTR1), have been disclosed as potential targets, but currently no specific inhibitor has entered the preclinical studies. Other parasite specific targets to be investigated involve redox

metabolism: enzymes which are essential for parasite survival under oxidative stress conditions include trypanothione reductase (TR) and trypanothione synthetase (TS), regulating the pool of the parasite unique dithiol trypanothione. Lastly, selective inhibition of trypanosome kinases has been proved to be a practicable route.

## *5.1 New Trypanothione Reductase Inhibitors and Other Deregulators of the Oxidative Status*

Trypanothione reductase (TR), as a key flavoenzyme catalyzing the reduction of the antioxidant dithiol trypanothione, protects trypanosomatids from the harmful oxidative stress generated by host cell defense systems. This attractive target is essential for parasite survival and does not present a corresponding homologue in humans, albeit being similar to function of the glutathione/glutathione reductase system. The discovery of agents able to inhibit TR activity is compulsory due to the *T. brucei* parasite enhanced sensitivity to oxidative stress and limited virulence after its inhibition. Among the different scaffolds of TR inhibitors (polyamines, peptides, benzimidazoles, nitroaryls, quinazolines), small molecules were preferred for their better pharmacokinetics and drug-like characteristics. A recent screening of a library of more than 3,000 compounds, using an optimized luminescence assay, led to identification of a new hit compound characterized by a new structural skeleton (1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one), inhibitory activity against *Tb*TR in the low micromolar range ($IC_{50} = 3.5 \pm 2.2$ μM), high solubility in PBS at pH 7.4, and with the ability to interact with a new pocket in the $TS_2$ (reduced tripanothione) binding site (Fig. 3a). This compound, 4-(((3-(8-(2-((1S,2S,5S)-6,6-dimethylbicyclo [3.1.1]heptan-2-yl)ethyl)-4-oxo-1-phenyl-1,3,8-triazaspiro[4.5]decan-3-yl)propyl) (methyl)amino)methyl)-4-hydroxypiperidine-1-carboximidamide **1**, exerted its biological potential only if concurrently substituted at all the nitrogen atoms as revealed by the results obtained after the design and synthesis of a large panel of diversely functionalized analogues. Moreover, this hit compound did not display inhibitory activity against its homolog glutathione reductase up to 50 μM, thus presenting a high selective toxicity for the parasite. From a kinetic point of view, it was shown to act as a competitive inhibitor with $TS_2$, without inducing conformational variations when bound. Lastly, in in vitro cultures of *T. brucei*, it increased the amount of reduced thiols in a dose-response manner ($IC_{50}$ value of $5.7 \pm 0.6$ μM), whereas the anti-proliferative effect was registered after 24 h treatment at $2.2 \pm 2.4$ μM.

Analysis of the X-ray crystallographic data revealed that it had two distinct binding sites in each *Tb*TR monomer. The first binding site, inside the wide trypanothione binding cavity, was characterized by hydrophobic interactions within 4 Å between the phenyl-triazaspiro scaffold and Trp21, Met113, and Tyr110. The flexible lateral chains (both the bicycle-heptane and the hydrophilic carboximidamide moieties) of the molecule established weaker interactions with Val53, Val58, Ile106, and Leu399. Intriguingly, the tertiary amino group along with

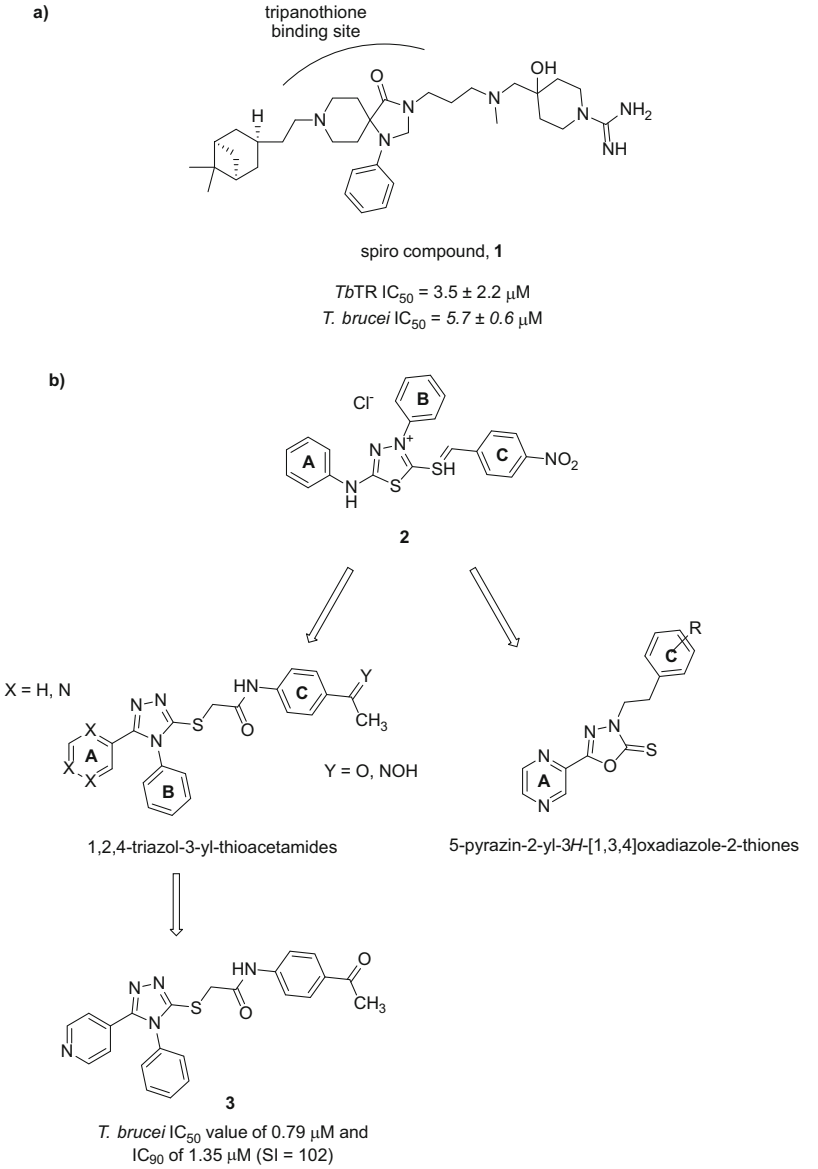


**Fig. 3** New trypanothione reductase inhibitors

the positive charge of other amino moieties, resembling the positive $TS_2$, contributed to this affinity by interacting with the overall negative charge of the cavity. Conversely, the other binding site was represented by a hydrophobic pocket near the interface of the *Tb*TR dimer, usually considered not important for any specific function because far away from the NADPH and the $TS_2$ binding cavities [58].

Recently, other two large series of nitrogen-containing heterocycles were investigated by Shaykoon et al. [59] with respect to α-difluoromethylornithine (DFMO) against *T. brucei*. The rational design of these compounds started from the evidence that the mesoionic 1,3,4-thiadiazolium-2-aminide (**2**) exhibited potent antitrypanosomal activity mediated by trypanothione reductase (TryR) inhibition in the nanomolar range (Fig. 3b). Keeping constant some pharmacophoric groups (a heterocyclic nucleus attached directly or through a spacer to two or three aromatic/heteroaromatic rings), 1,2,4-triazol-3-yl-thioacetamides and 5-pyrazin-2-yl-3*H*-[1,3,4]oxadiazole-2-thiones were designed with the aim to vary the heterocyclic ring (a) with pyrazine and pyridine, to replace the acetyl oxygen (Y) by hydroxylamino moiety, to change the substitution pattern on the phenyl ring B with electron-withdrawing (F) and donating ($CH_3$ or $OCH_3$) groups at *ortho*/*para* positions. The compounds were then tested for in vitro antitrypanosomal activity and cytotoxicity against rat myoblast L6 cell line.

The results allowed a clear assessment of SARs within the two scaffolds: among non-oxime 1,2,4-triazole compounds the presence at C5 of a pyridin-4-yl elicited inhibitory potency with respect to pyrazin-2-yl (e.g., compound **3** was more potent with $IC_{50}$ value of 0.79 μM and $IC_{90}$ of 1.35 μM than DFMO with $IC_{50} = 6.10$ μM and $IC_{90}$ of 8.66 μM). Conversely, the introduction of an oxime led to a slight decrement of the inhibitory activity, albeit preferring the pyridin-4-yl at C5. Within the 1,3,4-oxadiazole-2-thione series, the antitrypanosomal activity was in the micromolar range as follows: 4-$OCH_3$ > 4-$CH_3$ > 4-F at R. Other substitutions were unprofitable, except for derivatives characterized by 1,2,4-triazole pyrazines or pyridines containing a ketone or Mannich base with electron-donating groups. Overall, the tested compounds showed moderate cytotoxicity with selectivity indices ranging from 12 to 102 against L6 cells (DFMO displayed an SI of 12.17). Intriguingly, an exhaustive and comparative docking study was carried out toward ten pivotal *T. brucei* enzymes (rhodesain, TryR, sterol 14α-demethylase, pteridine reductase, purine nucleosidase, adenosine kinase, ornithine decarboxylase, UDP-galactose 4′-epimerase, dihydrofolate reductase, triphosphate isomerase) highlighting a discrete correlation among log ($IC_{50}$) of antitrypanosomal activity and their calculated $K_i$ values especially against TryR. More in detail, compound **3** did not display a strong affinity toward sterol 14α-demethylase, pteridine reductase, purine nucleosidase, ornithine decarboxylase, and UDP-galactose 4′-epimerase with respect to their co-crystallized ligands/inhibitors. Docking results, in terms of $\Delta G_b$, were comparable between **3** and the reference compounds against rhodesain, *T. brucei rhodesiense* adenosine kinase, triphosphate isomerase, and dihydrofolate reductase. This in silico investigation aimed to unravel the putative molecular targets mediating the antitrypanosomal activity and revealed a high binding affinity toward TryR (pdb: 2WP6) characterized by hydrogen bonds, hydrophobic and pi-sulfur interactions. These results were also in accord with the in vitro biological outcomes, being triazole derivatives more promising than oxadiazole derivatives. Compound **3**, overlapping with the triazole and the aromatic rings the reference mesoionic compound, occupied the binding site establishing H-bond interactions with Glu18 and several hydrophobic interactions with Leu17, Trp21, Tyr110, and Met113.

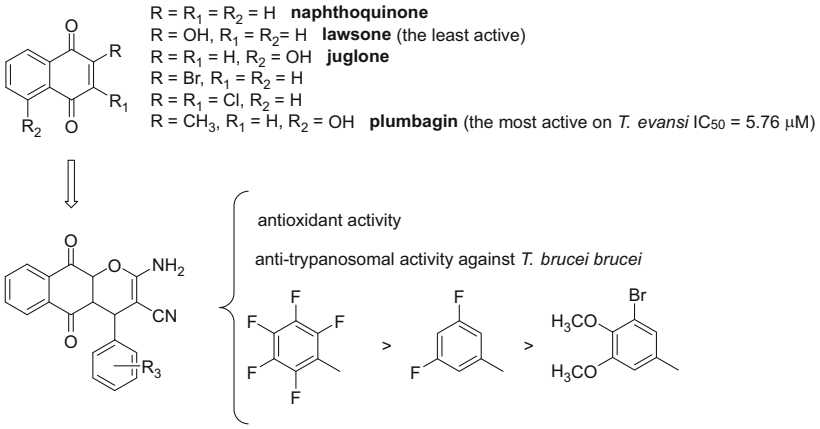


**Fig. 4** Naphthoquinone derivatives as antitrypanosomal agents

Another class of redox active compounds is represented by natural naphthoquinone derivatives (lapachol, atovaquone, juglone, plumbagin, lawsone), which were shown to induce the generation of ROS, hampering the normal cell functions. Recently, papers dealing with naphthoquinones focused on the antitrypanosomal activity against *T. brucei* and *T. evansi*. The first series explored simple naphthoquinones from synthetic and natural origin characterized by $IC_{50}$ values in the low micromolar range and the ability to induce an apoptotic-like mechanism in an axenic culture of *T. evansi*, by the ROS generation (Fig. 4). Compounds without an OH as $R_2$ were less active, being plumbagin the most potent as parasite growth inhibitor and ROS releaser [60].

Successively, the fusion of the lawsone scaffold with substituted phenylpyrans provided new active compounds against *Trypanosoma brucei* and other parasites with single digit micromolar $EC_{50}$ values. In addition, antioxidant activities were observed for the bromophenyl derivatives as $R_3$ with respect to ascorbic acid in the DPPH test, and their redox behavior was studied by cyclic voltammetry [61].

## *5.2 Nitro(Hetero)Cycle-Based Inhibitors of Nitro-Reductase*

Nitro(hetero)aryl compounds are the most populated group of antikinetoplastid (and antibacterial) molecules following the therapeutic and non-genotoxic efficacy of fexinidazole, nifurtimox, and benznidazole for the treatment of HAT. After oral administration, they undergo a bioactivation step into various reactive and electrophilic metabolites interacting covalently with cellular biocomponents. In *Trypanosoma* the reduction step involves type 1 nitro-reductases (NTRs), which are not present in mammals, thus representing a selective target.

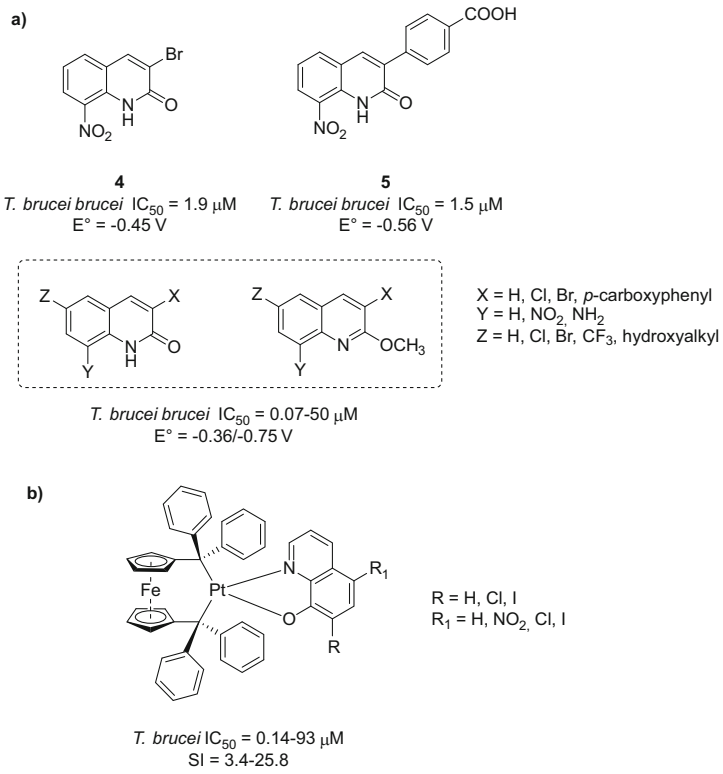


**Fig. 5** Nitro-reductase inhibitors

In the last years, the 8-nitroquinoline scaffold was largely explored in pharmacomodulation studies to assess the importance of a keto group at C2 (8-nitroquinolin-2(1*H*)-one), an intramolecular H-bond between the nitro group and the lactam function (**4**), and a substituent at C3 (halogen or *p*-COOH aryl, **5**) in order to be bioactivated by *T. b. brucei* type 1 NTRs in the low micromolar range (Fig. 5a). To enlarge this scaffold, new compounds were proposed by means of electron-withdrawing groups at C6 (halogens, $CF_3$, alkyl-alcohol) with or without $NO_2$ at C8 to facilitate bioactivation by nitro-reductases. All the compounds were first evaluated for their redox potential (couple $RNO_2/RNO_2^{\bullet -}$) in electrochemical studies. Cyclic voltammetry revealed redox potentials between −0.36 and −0.75V/NHE in DMSO, suggesting that a bromine atom or a trifluoromethyl group at C6 led to a better increase of E° than at C3. Concurrent presence of bromine at C3 and C6 induced a slight increase of E°. Moreover, cytotoxicity data of all compounds, assessed on the human HepG2 cell line, ranged from $CC_{50} = 17$ μM to $CC_{50} > 100$ μM, being more toxic the nitro-compounds with respect to the 8-unsubstituted derivatives. Less toxicity was also attributed to a *p*-carboxyphenyl functionality at C3. All the compounds were further screened against *T. b. brucei* trypomastigotes using as reference compounds suramin, eflornithine, and

fexinidazole. $EC_{50}$ values of some derivatives were between 12 and 200 nM and characterized by good selectivity indices (200–1,508). A nitro substituent at C8 was essential as well as a bromine at C6 (with respect to chlorine and trifluoromethyl). Finally, the three most promising derivatives were screened for microsomal stability, human albumin binding, and PAMPA assays along with aqueous solubility at physiological pH, mutagenic or genotoxic potential, in vivo tolerability, and inhibitory activity in *T. b. brucei* strains overexpressing the type 1 trypanosomal NTR [62].

The presence and the impact on the nitro-reductase inhibition exerted by the quinoline nucleus, with particular attention to the free OH at C8 and the N1, were further demonstrated when this scaffold was used as a chelating agent for new Pd-ferrocenyl compounds (Fig. 5b). Albeit maintaining a submicromolar/micromolar $IC_{50}$ values against *T. brucei* and good selectivity over mammalian macrophages (SI = 4–102), being more potent than nifurtimox, they were scarce deregulators of the cell thiol-redox balance. This behavior was limited despite the concurrent presence of the ferrocenyl moiety, which is known to be subjected to redox processes. The putative mechanism of action seemed to be oriented toward the interaction with the DNA as demonstrated by the partial displacement of ethidium bromide [63].

## *5.3 New Pteridine Reductase 1 Inhibitors*

The well-known compounds targeting dihydrofolate reductase (antifolates) are not effective, if used alone, against trypanosomatid parasites due to a metabolic bypass involving the expression of pteridine reductase 1. Some combined strategies have been proposed with monocyclic and bicyclic compounds acting as substrate-competitive inhibitors of dihydronicotinamide adenine dinucleotide phosphate (NADPH)-dependent pteridine reductase 1 (PTR1). Indeed, trypanosomatids and *Leishmania* parasites cannot biosynthesize endogenously pterin nucleus [64, 65], thus depending on the host metabolism for this pathway. Once absorbed, biopterins are reduced to dihydrobiopterin and tetrahydrobiopterin by PTR1 in a two-step process. These two cofactors are essential for parasite's growth and their lack brings to truncated cytokinesis, morphological changes, and death [66]. The target validation of this parasite enzyme came from the critical role exerted by NAPDH in the substrate recognition, the determination of its absence in humans, and the consequent possibility to limit side effects after its selective inhibition [67]. In the literature, different scaffolds, such as 2,4-diaminopteridines, quinazolines, 2,4-diaminopyrrolopyrimidines, 2-aminothiadiazoles, 2-aminobenzimidazoles, 2-aminobenzothiazoles, (1,6-dihydro)triazines, and chromen−/chroman-4-ones, have been recognized to bind to and inhibit the biopterin-binding site.

More than 60 crystal structures of *Tb*PTR1–inhibitor complexes have been deposited in the Protein Data Bank (PDB) [68] and starting from the four solved crystal structures of *Trypanosoma brucei* pteridine reductase 1 (*Tb*PTR1) in

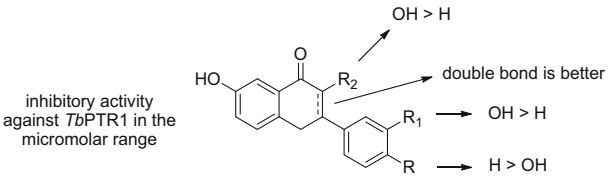


**Fig. 6** Flavanones as pteridine reductase 1 inhibitors

complex with three flavonols and one flavanone, Di Pisa et al. provided a further development of this class of compounds keeping constant the chroman-4-one nucleus and modifying specific positions and unsaturations. Collectively, all these compounds were micromolar inhibitors ($EC_{50}$ values) of the bloodstream form of *T. brucei*, but they did not display any evident cytotoxic effect against THP-1 cells (with selectivity index higher than 7). The in vitro inhibition data suggested an involvement of PTR1 inhibition as reported in Fig. 6 [69].

These flavanones and their corresponding flavanols were then studied in silico (docking studies and molecular dynamics) for their binding modes with the proposed target [70]. Data determined at 1.70 Å resolution for the complex involving one of these compounds revealed that the chroman-4-one moiety bound to *Tb*PTR1 unraveling the factors responsible for the differential efficacy of this small library of compounds and the impact of the cofactor NADPH in the modulation of such an inhibitory activity. First, the chroman-4-one moiety was engaged in a π-sandwich between the nicotinamide of $NADP^+$ and Phe97, whereas O1 pointed toward Asp161 and Tyr174. In addition, the OH at C6 formed two H-bonds: one stronger with $NADP^+$ and one weaker with Ser95. The carbonyl group at C4 is H-bound to Arg14 and connected to the oxygen of the cofactor through a water molecule. The phenol ring at C2 is oriented in a hydrophobic pocket (Met163, Val206, Leu209, Met213, Trp221, and Leu263) and made stacking interactions with Trp221 and a water-mediated bond with Asp161. Despite the soaking procedure was carried out on the racemic mixture, only the *R*-enantiomer has been registered in the active site cavity. Further advanced computational technique and MM/GBSA studies using the tetrameric *Trypanosoma brucei* pteridine reductase 1 (*Tb*PTR1, UNIPROT entry no: O76290, PDB code 5K6A) calculated a high binding energy of −49.0507 Kcal/mol for this compound and that Arg14, Ser95, Phe97 residues to contribute more to the binding and stabilization dynamics at the *Tb*PTR1 pocket. Once again, the chroman-4-one moiety was responsible for conspicuous electrostatic energy contributions within the deepest part of the active site (∼15 Å of depth), highlighting the promising development of more expanded scaffolds (e.g., tricyclic-based compounds) able to be accommodated for the entire volume in the *Tb*PTR1 catalytic pocket.

As an expansion of the pharmacophoric 2,4-diaminopyrimidine moiety present in classical DHFR inhibitors, 2,4-diaminopyrimido[4,5-*b*]indol-6-ol was found to be efficacious in blocking competitively the *Tb*PTR1 activity in vitro with a $K_i$ in the low micromolar range. The binding mode was attributed to the formation of a ternary

complex with *Tb*PTR1 and the cofactor, adopting a substrate-like orientation inside the biopterin-binding pocket and maximizing the binding hydrophobic and hydrogen-bond contributions in all four subunits of the tetramer [71]. As seen for chroman-4-ones, the tricyclic aromatic system was engaged in the π-sandwich formed by Phe97 and the cofactor. The amine moieties at C2 and C4 of the pyrimidine established direct or water-mediated interactions with the β-phosphate and could form hydrogen bonds to Ser95 and the ribose hydroxyl of the NADPH. Moreover, the third aromatic ring was successfully accommodated in a hydrophobic pocket (Val206, Leu208, Pro210, Met213, Trp221) of the active site of *Tb*PTR1. Two residues of this hydrophobic pocket were at the edge of another hydrophobic pocket (Met163, Cys168, Leu263) which could be further exploited expanding this molecular scaffold.

## *5.4 Selective Methionyl-tRNA Synthetase Inhibitors*

Methionyl-tRNA synthetase of *T. brucei* (*Tb*MetRS, EC 6.1.1.10) has been widely in vitro and in vivo validated as a new drug target for the treatment of HAT, after the discovery that it is essential for parasite proliferation and its inhibition resulted in trypanocidal activity. Indeed, as a member of the aminoacyl-tRNA synthetase family, this enzyme catalyzes an important role in protein synthesis and production of methionyl-tRNA in a two-step procedure, allowing the incorporation of methionine into nascent proteins during translation.

Inhibitors must be designed to be also selective for this isoform and able to cross the BBB. Evidence from bacterial and *Leishmania* MetRS inhibitors paved the way to compound **6** in Fig. 7a, whose crystal structure in complex with *Tb*MetRS was solved. Generally, they share little chemical diversity and a common binding to two well-characterized enzyme pockets highly conserved among kinetoplastid MetRS enzymes. The 3,5-dichlorophenyl moiety occupied the binding pocket for methionine, whereas the opposite benzimidazole moiety was bound to an auxiliary pocket which was opened by the presence of the inhibitor. The central and linear 1,3-propyldiamino linker connected the two cycles and was modified into closed rings (pyrimidine) keeping constant the 1,3,5- or 1,2,4-substitution pattern on the aromatic ring.

To further expand the SAR studies, a first new series of compounds were explored modifying the pyrimidine ring with a linear linker of $\leq$6 atoms or incorporating rigid structures (1,3-dihydro-imidazol-2-one, triazaheterocycles). Then, new substitution patterns were focused on both the two (hetero)aryl sides obtaining the results shown in Fig. 7b, c. The most active compounds (**7** and **8**) not only inhibited the enzyme in the low nanomolar range, but also blocked the parasite proliferation at low concentrations. The *Tb*MetRS enzyme inhibition was similar to the previous solved crystal of compound in Fig. 7a. The substituents inserted on the benzimidazole nucleus were driven by the space limitation of the auxiliary binding pocket. All inhibitors displayed low toxicity with $CC_{50}$s $\geq$ 20 μM against

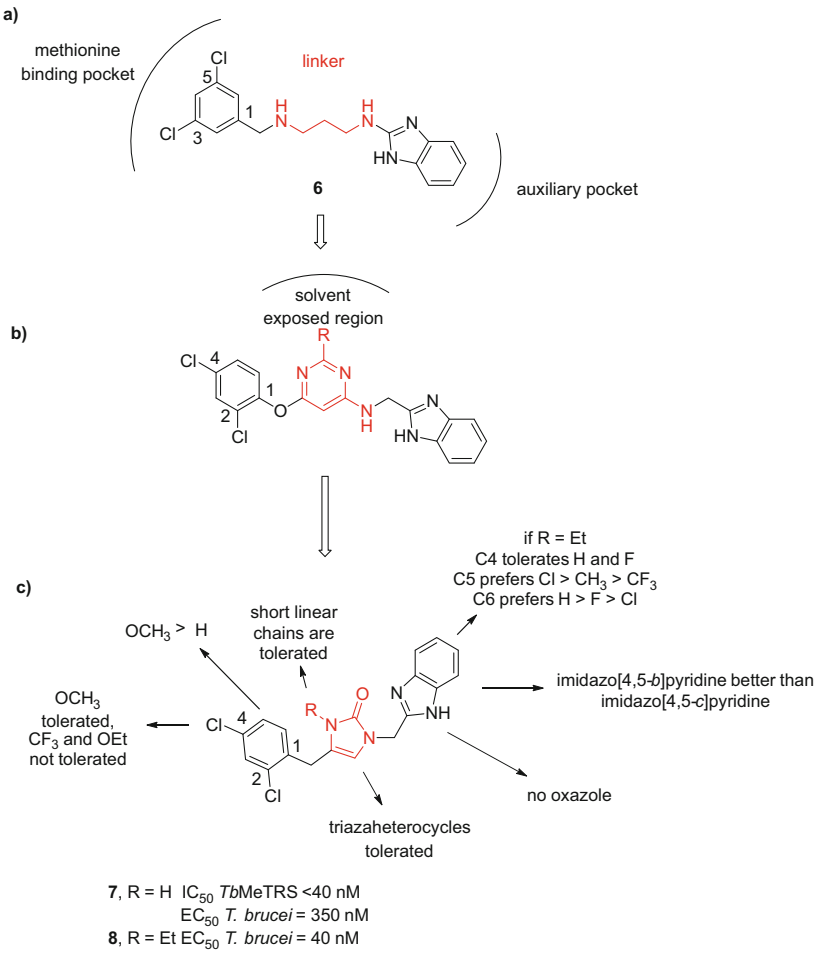


**Fig. 7** Methionyl-tRNA synthetase inhibitors

mammalian CRL-8155 and HepG2 cells, good selectivity over human mitochondrial MetRS, but non-satisfactory BBB penetration properties [72].

## 5.5 *Phthalazinone Derivatives as Novel* T. brucei *Phosphodiesterase Inhibitors*

Following the efforts sustained by the European consortium "Parasite-Specific Cyclic Nucleotide Phosphodiesterase Inhibitors To Target Neglected Parasitic Diseases," parasitic 3′,5′-cyclic nucleotide phosphodiesterases (PDEs) were proposed as genetically important molecular targets. They are characterized by the P-pocket in

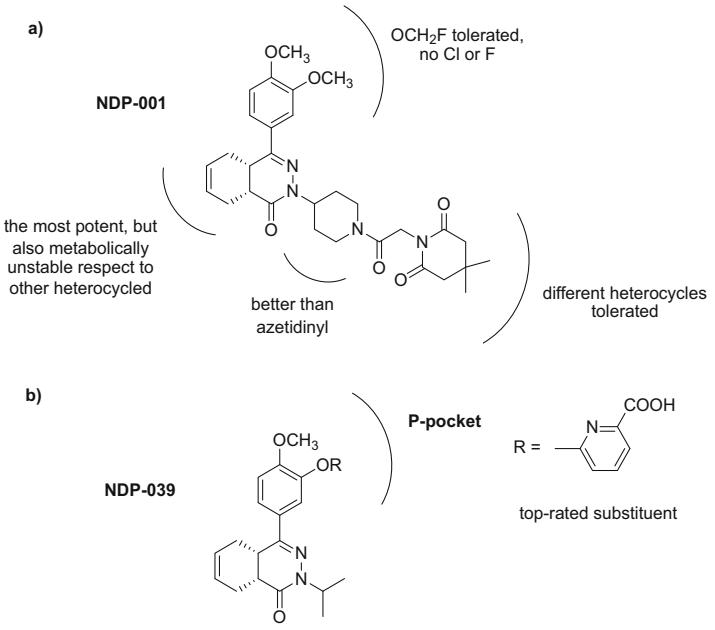


**Fig. 8** Phthalazinone derivatives as *T. brucei* phosphodiesterase inhibitors

the substrate binding site. This subpocket potentially could impart selectivity, limiting the inhibition of the human off-targets (hPDEs) and the consequent side effects (nausea, emesis, TNF-α inhibition). *T. brucei* genome encodes for five trypanosomal cyclic nucleotide phosphodiesterases, among which *Tbr*PDEB1 and *Tbr*PDEB2 are the most studied and essential for parasite virulence [73]. Moreover, their silencing or reduced expression led to distortions of the cell cycle, parasite cell death, and improved animal survival after parasite infection. These two paralogues share 88% structural identity of the catalytic domain and simultaneous inhibition is possible as reported for the phenylpyridazinone compound **NPD-001** shown in Fig. 8 ($IC_{50}$ *Tbr*PDEB1 = 12.0 nM; $IC_{50}$ *TBr*PDEB2 = 12.4 nM). This lead compound was discovered after a high throughput screening campaign by de Koning et al. (2012) [74] due to its early identification as *h*PDE4 inhibitor. It interacted with the P-pocket by the rigid biphenyl glycinamide installed on the tetrahydrophthalazinone nucleus. Despite the encouraging in vitro results ($IC_{50}$ = 6.3 μM) and selectivity over human isoforms, its low metabolic stability did not allow to reach a sufficient efficacy in an in vivo murine model infected with *T. brucei* trypanosomes.

Starting from this information, wide structural modifications and in silico predicting tools to improve metabolic stability were used to maintain high potency inhibitors against *Tbr*PDEB1 and *T. brucei* [75]. A first panel of 62 derivatives were elegantly synthesized and tested through a phenotypic screening against *T. brucei*, MRC5 for cytotoxicity, and on the isolated catalytic domains of *Tbr*PDEB1 and

*h*PDE4 for affinity and selectivity. All the compounds were active against the target enzyme, but only few were unable to permeate the parasite membrane to exert their anti-proliferative effects.

According to the metabolic instability of the parent compound, the 3,4-di$OCH_3$ moiety, the unsaturated cycle of the phthalazinone, the amide portion of the linker and the heterocycle in the latera chain were modified. As reported in Fig. 8, the 3,4-dimethoxy moiety is the most favorable as well as the $OCHF_2$ substitution. Poorer results were obtained with Cl or F. The unsaturated phthalazinone is the most preferred, but also metabolically unstable. Its importance was demonstrated by crystallographic structures in which it occupies an area near the metal site (magnesium and zinc) engaging the hydrophobic clamp with residues Val840 and Phe877 and an H-bond interaction involving the strictly conserved Gln874 within the phosphodiesterase family. As a linker, a piperidine is clearly favored over an azetidine, whereas the heterocycle attached to the linker could impact differently on inhibitory potency and cytotoxicity (six-membered better than a five-membered ring) and depended on the substitution pattern on the opposite aromatic ring. Collectively, these compounds displayed low selectivity toward the human isoform *h*PDE4, because of the lacking interaction with the P-pocket.

The same research group explored this possibility based on the crystallographic structure of another tetrahydrophthalazinone analogue **NPD-039**, which has a rigid biphenyl glycinamide on the phenyl ring addressing the P-pocket in the substrate binding site ($K_i$ *Tbr*PDEB1 = 0.1 μM; $K_i$ *h*PDE4 = 1.9 μM) (Fig. 8) [76]. In order to provide much flexibility, they introduced a diaryl ether function inserting commercially available heteroaromatic moieties to explore their influence and a largely functionalized pyrid-2-yl group in C3. Most of the compounds were suggested by a computer-aided design and were tested to study the interaction with *Tbr*PDEB1, cytotoxicity for MRC-5 cells, and in vitro efficacy against *T. brucei* [77]. The 5-membered and 6-membered rings furnished low micromolar inhibition of *Tbr*PDEB1 (especially pyrazine and pyridine), whereas larger and bulky fused rings showed no inhibition up to 10 μM to be accommodated in the P-pocket. The introduction of substituents on the pyridine-2-yl ring gave few improvements, except for a carboxylic group at the sixth position. These compounds also showed inhibitory activity in vitro in the range of 7.9–25 μM and low cytotoxicity on mammalian cells ($CC_{50} > 64$ μM).

## 5.6 *Advances in Trypanosome Peptidases: The Case Study of Cysteine Protease Rhodesain Inhibitors*

Five major classes of proteolytic enzymes (peptidase) have been discovered after purification or detection within the completed Genome Projects [55]. Rhodesain (*Tbr*CATL, also known as brucipain or trypanopain) is a cathepsin L (CATL)-like protease mainly expressed in *T. brucei rhodesiense* and located in the lysosomes in

all parasite life-cycle stages. Similar to cruzipain, the mature TbrCATL is a single chain of 215 peptidic residues, with a conserved catalytic triad (Cys/His/Asn). The first crystallographic structures with the vinyl sulfone inhibitors **K777**, at a resolution of 1.65 Å (PDB 2P7U), indicated to be superimposable to the structure of cruzipain, disregarding the substitution of Glu with Ala in the S2 subsite which leads to a different substrate recognition. The role of *Tbr*CATL involves the lysosomal degradation of both protozoan and host proteins after being released from bloodstream trypomastigotes. It is important for both *T. brucei* subspecies in terms of survival, infectivity, and CNS penetration. Moreover, it is responsible for the harmful stimulation of protease activated receptors in the brain endothelial cells, for escaping the immune system by stimulating the variant surface glycoproteins (VSGs) of the trypanosome coat and the degradation of immunoglobulins, and for the onset of the cardiac pathology by changes in the sarcoplasmic reticulum function. Lastly, it has been recognized as enhancer of suramin toxicity for the parasite and can be administered with curcumin to exert a synergistic effect [78].

Starting from the structure of the covalent inhibitor **K777**, characterized by a vinyl sulfone moiety as a Michael acceptor occupying the P1′ site, changes were performed on the final nitrogen-containing heterocycle (quinoline) and the phenethyl group as a lateral chain [79]. SAR studies and in silico covalent docking evaluation confirmed that the phenethyl side chain could be hardly replaced by benzyl, propyl, butyl, isobutyl, or H and must be retained in the structure due to the occupation of the P1 site of the enzyme. The quinoline ring could be substituted to flexible partially saturated, methylated dihydroquinoline, methylated tetrahydroquinoline, or tetrahydroquinoline ring, keeping constant the inhibitory activity against the enzyme, but changing the anti-proliferative effects of *T. brucei*. When this ring is converted to monocyclic heterocycles (pyridine, 1,4-dimethyl-1*H*-imidazole, 1,3-thiazolidine) the enzymatic inhibition could be conserved, but the activity against *T. brucei* is weaker. Only nitroaromatic-based compounds display equipotency against *T. brucei* leading to the possibility of dual-acting compounds (inhibition of *Tbr*CATL and trypanocidal action of the nitroaromatic moiety) (Fig. 9a).

Other two papers, from the same research group, explored the importance of a Michael acceptor moiety for the design of covalent inhibitors of rhodesain. The first paper deals with the substitution of the vinyl sulfone with peptidomimetic α,-β-unsaturated (vinyl) esters in which the phenethyl lateral chain was maintained, but the terminal ring was a benzodiazepine (BDZ) structure as a β-turn mimetic [80]. This functionalization could also provide optimal oral bioavailability and tolerability as reported for their parent compounds. The bulkiness of the ester group should be kept limited for a proper inhibition of the enzyme and the interaction with the S1 site (Fig. 9b), being the benzyl group like a butyl one. Conversely, these compounds displayed low selectivity with respect to cathepsin L, a human cysteine protease, and in the cell-based tests against *T. brucei brucei* the antitrypanosomal activity followed the higher lipophilicity of the R group. The second paper took advantage of a vinylketone as a Michael acceptor (warhead) and the introduction of fluoro and methyl substituents on the aromatic rings (P2 and P3) (Fig. 9c). This

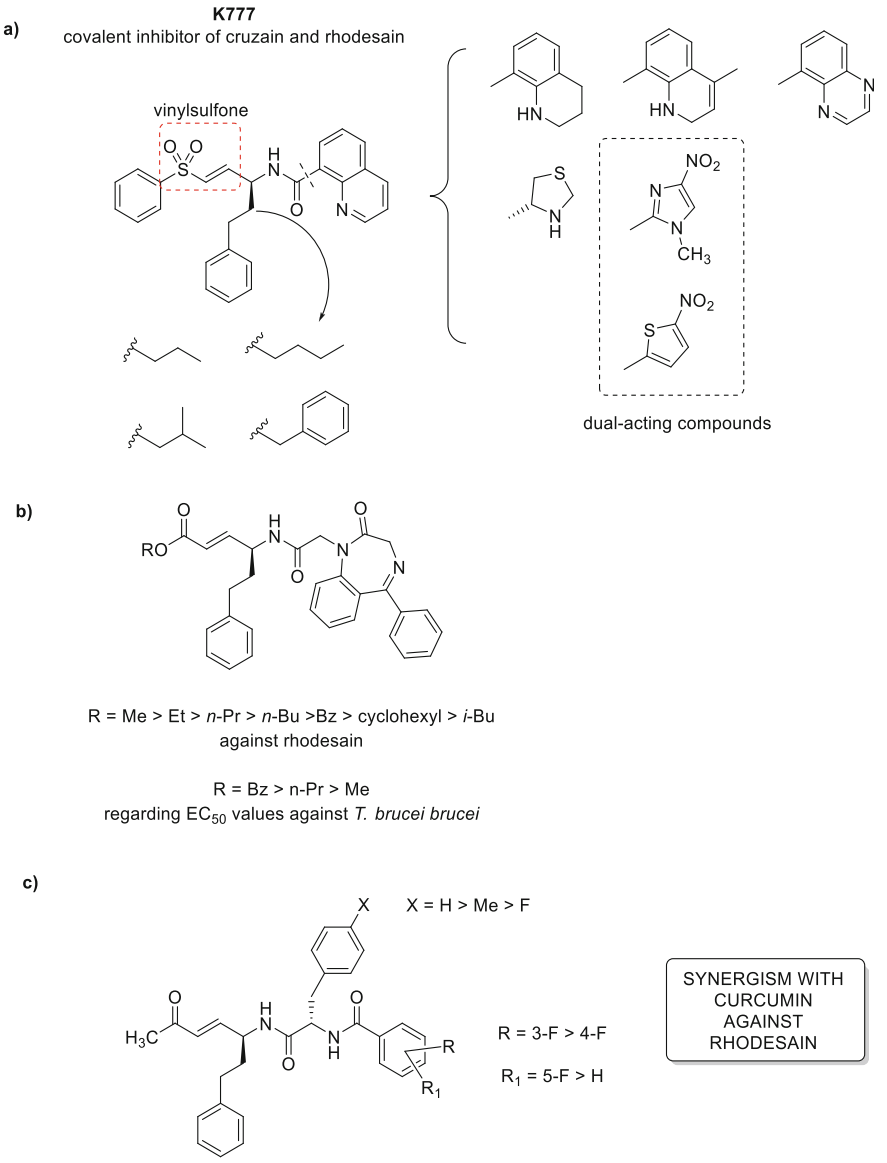


**Fig. 9** Rhodesain inhibitors

pattern led to a promising inhibition of rhodesain and a marked selectivity over human cathepsin L. On the other hand, the presence of a cyclohexyl methyl group as P2 limited both these factors, despite their trypanocidal activity was comparable to the former in the cell-based assay [81].

## 6 Conclusion

The search for new compounds against *T. brucei* infection proceed slowly in the last years despite the presence and activity of international consortia as also demonstrated in Fig. 10, where the number of published papers on Pubmed related to this protozoan (using the keyword: *Trypanosoma brucei*) decreased in the past two years.

The attention was mainly devoted to the differences exploitable in terms of enzymes/proteins important for the parasite growth and virulence, but absent or non-targetable in the host. We have explored different emerging targets highlighting the SARs in the most recent studies. Most of these articles dealt with the inhibition of *T. brucei* species, without discriminating between the two subspecies *gambiense* and *rhodiense*. Species of veterinary interest (*T. brucei brucei*, *T. evansi*) are also emerging as important topics, but no in vivo studies were recently presented.

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*Ethical Approval:* This article does not contain any studies with human participants performed by any of the authors.

*Conflict of Interest:* The authors declare that they have no conflict of interest.

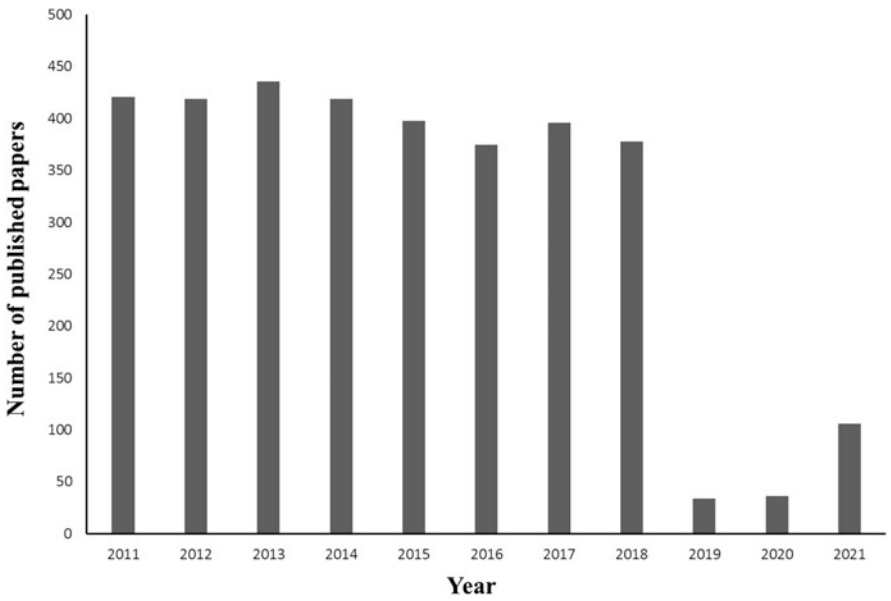


**Fig. 10** Number of published articles on *T. brucei* within the 2011–2021 range

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# Polyamine and Trypanothione Pathways as Targets for Novel Antileishmanial Drugs


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**Abstract** Leishmaniasis is an infectious disease classified by WHO as one of the neglected tropical diseases. Due to the lack of human vaccines, chemotherapeutic agents represent the only strategy for disease combat. However, the current treatment is marked by variable efficacy, high toxicity, and high cost. Thus, the search for more efficient antileishmanial agents becomes urgent. Several studies carrying out the discovery or development of potent inhibitors of key enzymes of *Leishmania* metabolism have demonstrated promising results. The polyamine and trypanothione pathways are essential for parasite survival and pathogenesis. Polyamine synthesis allows parasite growth and influences infectivity. Moreover, the final product of the polyamine pathway spermidine is required for the synthesis of trypanothione, a scavenger of reactive oxygen and nitrogen species, which is essential for the maintenance of *Leishmania* redox balance. In the present chapter, the advances in the use of synthetic and natural inhibitors of the polyamine and trypanothione pathways from *Leishmania* are discussed.

**Keywords** Antileishmanial activity, Enzyme targets, Leishmaniasis, Poliamine metabolism, Trypanothione metabolism

## 1 Introduction

Leishmaniasis is an infectious parasitic disease caused by several protozoa species belonging to the *Leishmania* genus, which is transmitted by female phlebotomine sand flies. It is listed as one of the tropical neglected diseases that afflicts several populations worldwide, especially those located in tropical and subtropical areas. Leishmaniasis occupies the ninth position among the most prevalent infectious diseases worldly [1]. In addition, it is considered the second leading cause of world death by parasitic infection, ranking behind malaria only. The clinical manifestations of the disease are associated with the parasite species as well as the patient's immune response. Clinically, the disease is classified as tegumentary (TL) and visceral (VL) leishmaniasis. The first one may evolve to cutaneous (CL), mucocutaneous (MCL), diffuse (DL), or disseminated cutaneous (DCL) leishmaniasis. VL consists in the most lethal form of the disease due to the extensive damage to internal organs, such as spleen, liver, and bone marrow. This form is responsible for 20,000 to 40,000 world deaths annually [2, 3].

It is estimated that approximately 0.2 to 0.4 million cases of VL and 0.7 to 1.2 million cases of TL occur annually. According to the World Health Organization, over 90% of VL cases are reported in six countries: India, Bangladesh, Sudan, South Sudan, Ethiopia, and Brazil. TL is more widely distributed; 70–75% of the estimated global incidence occurs in Afghanistan, Algeria, Iran, Syria, Ethiopia, North Sudan, Costa Rica, Peru, Colombia, and Brazil [3]. It is noteworthy that Brazil has a high burden of both clinical forms of leishmaniasis [4].

In recent decades, there has been progress in the development of effective vaccines against leishmaniasis [5]. Indeed, some of them reached clinical trials, including the second generation vaccines LEISH-F1 (Phase II) and LEISH-F2 (Phase II) against CL, and LEISH-F3 (Phase I) against human VL [6]. In addition, ChAd63-KH, a third generation vaccine against VL, reached Phase I trial with promising results in healthy volunteers [7]. Nevertheless, there is no vaccine approved for human leishmaniasis so far. The current treatment consists in the use of chemotherapeutics, which are known to be highly toxic and expensive. Severe side effects, variable efficacy, and low accessibility in certain afflicted areas aggravate this scenario. In addition, some of these drugs have a long history of use (pentavalent antimony compounds have been used since 1940), which enabled the emergence of resistant parasite strains [8]. Thus, several efforts have been made to discover novel, more effective, and accessible antileishmanial agents. Many of them fomented by the DNDi (Drug for Neglected Diseases Institute) organization through its international programs and the redeLEISH Network, which is dedicated to share information on treatment, diagnosis, and development of clinical trial design for CL cases [9]. Currently, many antileishmanial substances are being directed toward the inhibition of specific and vital parasite targets, including metabolic pathways, organelles, membrane components, etc. [10]. Among those, *Leishmania* proteasome has been extensively studied as parasites are highly dependent on their protein quality control machinery to sustain rapid growth and division [11]. Remarkably, the chemical class of azabenzoxazoles proved to be active against trypanosomatid infections, including *L. donovani*, by proteasome inhibition [12].

Enzymes are extremely important for *Leishmania* pathogenesis, as they play key roles in the host/parasite interaction, including: (1) modulation of the host immune system; (2) invasion and destruction of host tissues; (3) migration, growth, and nutrient acquisition, which are essential for parasite survival and proliferation, required for the maintenance of infection. Furthermore, enzymes are considered important virulence factors [13]. Trypanosomatid protein kinases have been reported as promising targets for new drug candidates, since these enzymes act in several cellular processes. Interestingly, trypanosomatid protein kinases display certain differences from their mammalian counterparts, paving the way for the development of highly specific inhibitors [14]. Recently, a detailed mechanistic study revealed that a series of pyrazolopyrimidine analogues with proved anti-*L. donovani* activity in vivo act by inhibiting the cdc-2-related kinase 12 (CRK12), pointing at this enzyme as a validated drug target for VL [15].

The search for essential enzyme inhibitors has drawn attention to purine/pyrimidine salvage pathways and the use of nucleoside analogues against *Leishmania* infection [10]. The pyrimidine nucleoside analogue 5-fluorouracil and the purine nucleoside analogue azathioprine displayed important anti-intracellular *L. donovani* and *L. infantum* amastigotes activity with $IC_{50}$ values $<10$ μM. In addition, a series of 5′-Norcarbocyclic nucleoside analogues were successful in inhibiting growth of *L. mexicana* promastigotes [16]. Among *Leishmania* metabolic pathways, polyamine biosynthesis has been extensively studied as a therapeutic target for the discovery of new antileishmanial substances due to its role in parasite growth,

differentiation, and proliferation [17]. Moreover, the polyamine spermidine is a precursor of the biosynthesis of trypanothione, an important molecule associated with parasite protection against oxidative and nitrosative stresses. Trypanothione is able to scavenge reactive oxygen and nitrogen species, which are lethal to *Leishmania* [18, 19]. In this chapter, we discuss the current scenario of leishmaniasis treatment and address the recent advances on the discovery of new antileishmanial agents based on the inhibition of enzymes from the polyamine and trypanothione biosynthetic pathways.

## 2 Leishmaniasis Chemotherapy

The current scenario of leishmaniasis treatment was certainly improved in the last few decades. However, it is far from ideal since it still hampers on important issues, such as high toxicity, serious side effects, variable efficacy, and parasite resistance. In this topic, we focus on the most common therapeutic options available against the disease and their respective mechanisms of action.

The chemotherapeutic approach for leishmaniasis treatment depends on which country the patient is located, as well as his/her clinical condition and the parasite species involved in the infection. Several well-known factors can alter the efficacy of leishmaniasis treatment, such as: (1) parasite and host phenotype; (2) host's immune response and socioeconomic condition; (3) side effects, (4) emergence of resistant strains; (5) high diversity of pathogenic *Leishmania* species [20]. Currently, the most frequently used chemotherapy drugs against all clinical cases of leishmaniasis are pentavalent antimonials and amphotericin B (free and liposomal form). However, the therapeutic arsenal also includes pentamidine, paromomycin, and miltefosine. Combination therapy and immunotherapy are additional strategies to control the disease and the emergence of resistance [21].

Pentavalent antimonials have been prescribed for more than 70 years. These drugs are classified as prodrugs, since they are reduced intracellularly to the most active trivalent form [22]. The mechanism of action of the trivalent form includes the trypanothione reductase inhibition, followed by an exacerbated oxidative stress and parasite death [23, 24]. It is worth mentioning that the activity of the pentavalent form is reported, and it is attributed to inhibition of DNA type I topoisomerase and formation of complexes with ribonucleosides that affect purine transport [22]. Other mechanisms of action reported for antimonials include inhibition of macromolecules (proteins, DNA, and RNA) and ATP synthesis. The last one is probably due to the inhibition of glycolytic enzymes and β-oxidation of fatty acids, two of the three parasite's energy generation mechanisms [25, 26]. In addition, antimonials also play an immunomodulatory effect on infected macrophages that induce oxidative stress and parasite killing [27]. Antimonials are toxic and their use may cause hepatitis, pancreatitis and, mainly, cardiotoxicity. In addition, these drugs are not indicated for patients with HIV co-infection or during pregnancy. In Bihar (India), antimony-

resistant visceral leishmaniasis has been reported, which demonstrates the urgent need for new therapeutic strategies [28].

The second-line drug for the treatment of leishmaniasis is amphotericin B, an antifungal agent originally produced by *Streptomyces nodosus* [29]. Amphotericin B mechanism of action is attributed to its binding affinity to *Leishmania* plasma membrane ergosterol. However, this drug also binds to cholesterol from the host cell membrane, which is the main cause of side effects and toxicity [30]. Amphotericin B binding to sterols changes the membrane permeability and, consequently, leads to electrolyte imbalance and parasite death. Interestingly, the ability of amphotericin B to complex and sequester cholesterol from the host cell membrane inhibits *Leishmania* binding to the macrophage, decreasing infection. Thus, the anti-*Leishmania* effect of amphotericin B seems to be due to a reduction in both parasite ergosterol and host cell cholesterol levels [31, 32]. Moreover, amphotericin B acts by increasing a proinflammatory response and activating host cells to produce reactive nitrogen and oxygen species that are essential for disease control [33, 34]. Despite the high cure rates of amphotericin B, this drug has severe side effects, such as nephrotoxicity, hypokalemia, and myocarditis. The amphotericin B treatment is expensive, especially if the liposomal form is prescribed. In addition, patient hospitalization is needed in order to avoid complications during drug administration [28].

Pentamidine has been prescribed for the treatment of leishmaniasis for more than half a century. This aromatic diamidine was initially used against African trypanosomiasis, but it started showing results against cases of *Leishmania* infection resistant to antimony [29]. Pentamidine targets the polyamine pathway, as demonstrated by decreasing the levels of intracellular arginine, ornithine, and putrescine, in addition to inhibiting polyamine [35] and arginine transport [36]. Other mechanisms of action have been reported, such as inhibition of kDNA replication [37] and nucleoside triphosphate diphosphohydrolase 1 (NTPDase1) [38], and binding to DNA and ubiquitin [39]. Despite good results in leishmaniasis treatment, pentamidine has been associated with pancreatitis, hypoglycemia, and hypotension.

The aminoglycoside paromomycin is a broad-spectrum antibiotic produced by *Streptomyces rimosus* var. *paromomycinus* that displays antileishmanial activity. Indeed, this drug is effective for VL treatment, being introduced for this purpose in 2006. Despite promising results, the use of paromomycin as a monotherapy increases the risk of emergence of resistant parasites. Therefore, combination with other antileishmanial drugs is often recommended [40]. Regarding paromomycin effects against TL, most studies were conducted in the New World with variable results [20]. In spite of the variable efficacy against clinical isolates and reference strains, paromomycin displays anti-intracellular amastigote activity against dermotropic *Leishmania* spp. [41]. The mechanism by which paromomycin eliminates parasites includes interference with both RNA and protein synthesis, as well as plasma membrane permeability [42–44].

Miltefosine is an alkyl phospholipid drug originally developed as an antineoplastic agent [29]. It is the first oral drug effective against leishmaniasis, especially the visceral form and post-kala-azar dermal leishmaniasis (PKDL). The drug was

released in the early 2000s and showed high efficacy in Asia. However, in some regions, the use of miltefosine for so many years has decreased its effectiveness, leading to the discontinuation of treatment. In the New World, miltefosine showed promising results against TL. However, it was demonstrated that geographical area and dermotropic *Leishmania* strains influence miltefosine effectiveness [20]. Moreover, the high incidence of liver, gastrointestinal and kidney toxicities, in addition to teratogenicity demonstrates that miltefosine prescription must be closely monitored. Miltefosine mode of action includes inhibition of lipid metabolism [45], plasma membrane permeabilization [46, 47], $Ca^{2+}$ release [29, 48], cytochrome *c* oxidase inhibition [49], increase in reactive oxygen species [50], and inhibition of RNA synthesis [51]. Finally, miltefosine is able to induce the activation of a Th1-type immune response that is associated with the control of infection [52].

## 3 Polyamine Pathway

Polyamines are ubiquitous aliphatic polycations essential to all cells. *Leishmania* parasites use polyamines in the biosynthesis of macromolecules that promote cell growth, proliferation, and differentiation. *Leishmania* amastigotes and promastigotes are capable of uptaking polyamines from the environment through specific transporters [53]. However, polyamine transport is saturable, requires metabolic energy, and is sensitive to temperature changes [54, 55]; therefore, cells are equipped with enzymatic machinery for polyamine biosynthesis. In addition, polyamine transporters have been studied as potential targets for leishmaniasis treatment, highlighting the relevance of the polyamine pathway for the development of new therapeutic strategies [56].

The first step in the biosynthesis of polyamines is the conversion of L-arginine into L-ornithine and urea, which is catalyzed by arginase (ARG). Then, L-ornithine is decarboxylated into putrescine (1,4-diaminobutane) by ornithine decarboxylase (ODC), a pyridoxal-5′-phosphate (PLP)-dependent enzyme. In parallel, S-adenosylmethionine decarboxylase (AdoMetDC) catalyzes the decarboxylation of adenosylmethionine (dcAdoMet), a direct precursor of spermidine, through the donation of an aminopropyl group. Then, spermidine synthase (SpdS) catalyzes the ligation of this aminopropyl group from AdoMet to putrescine, forming 5′-deoxy-5′-methylthioadenosine (MTA) and the polyamine spermidine. Figure 1 illustrates the *Leishmania* polyamine pathway. The inhibition of polyamine biosynthesis has been reported as a promising strategy for the control of infection. In fact, each enzyme may represent a potential target for the development of new drug candidates [57]. In the next sections, we discuss the enzymes that compose the polyamine pathway and their respective inhibitors.

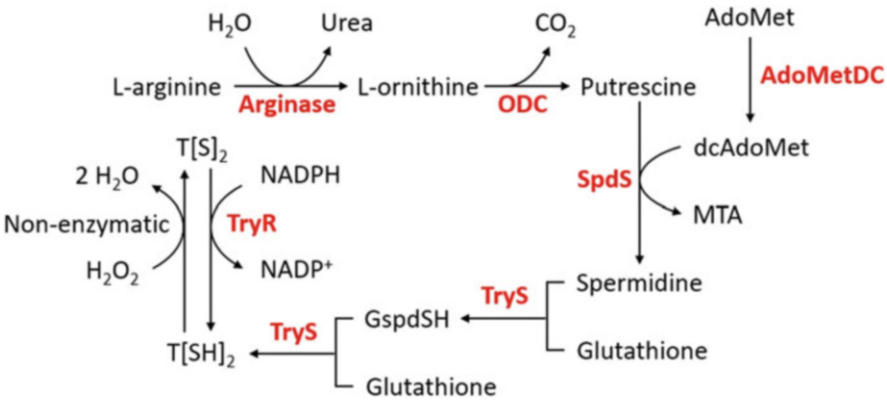



**Fig. 1** Polyamine and trypanothione pathways in *Leishmania*

## *3.1 Arginase*

As mentioned earlier, the metalloenzyme ARG regulates the flow of L-ornithine to the biosynthesis of polyamines [58] and thus is a key enzyme for the establishment of the disease. A remarkable work by Roberts et al. [59] showed that an ARG knockout strain of *Leishmania mexicana* is unable to grow and proliferate, revealing that ARG is essential for parasite viability and thus constitutes a potential therapeutic target. The lethality of the ARG mutant could be circumvented by supplementation of either high concentrations of ornithine or spermidine or low concentrations of putrescine, suggesting that the sole function of ARG in *Leishmania* is to provide precursors for the biosynthesis of polyamines [59].

Several studies have been conducted to discover novel inhibitors of *Leishmania* ARG from natural or synthetic origins. Among the natural inhibitors, those belonging to the class of phenolics, specifically flavonoids, are certainly the most relevant ones. The flavonoid orientin (luteolin-8-C-glucoside), the major compound in the ethyl acetate fraction of *Cecropia pachystachya*, was identified as an inhibitor of *Leishmania amazonensis* ARG (LaARG) with 50% enzymatic inhibition concentration ($IC_{50}$) of 15.9 μM [60]. Other flavonoids displaying promising LaARG inhibitory activity include quercetin ($IC_{50}$ = 4.3 ± 0.03 μM), quercitrin ($IC_{50}$ = 10 ± 0.08 μM), fisetin ($IC_{50}$ = 1.49 ± 0.3 μM), and luteolin ($IC_{50}$ = 9 ± 1 μM). In addition, naturally occurring quercetin derivatives such as rhamnetin, rutin, avicularin, guaijaverin, hyperoside, quercetin-3-*O*-glucuronide, and taxifolin inhibited LaARG activity with $IC_{50}$ values ranging from 1.6 ± 1 to 10.4 ± 0.8 μM [61]. The relevance of flavonoids as potential ARG inhibitors was revealed by in silico studies. It seems consistent that the presence of a catechol group (1,2-dihydroxybenzene) plays a pivotal role in the inhibitory activity. Docking analysis showed that the catechol group interacts with amino acid residues that participate in the formation of a $Mn_A^{+2}$-$Mn_B^{+2}$ metal bridge at the enzyme's catalytic

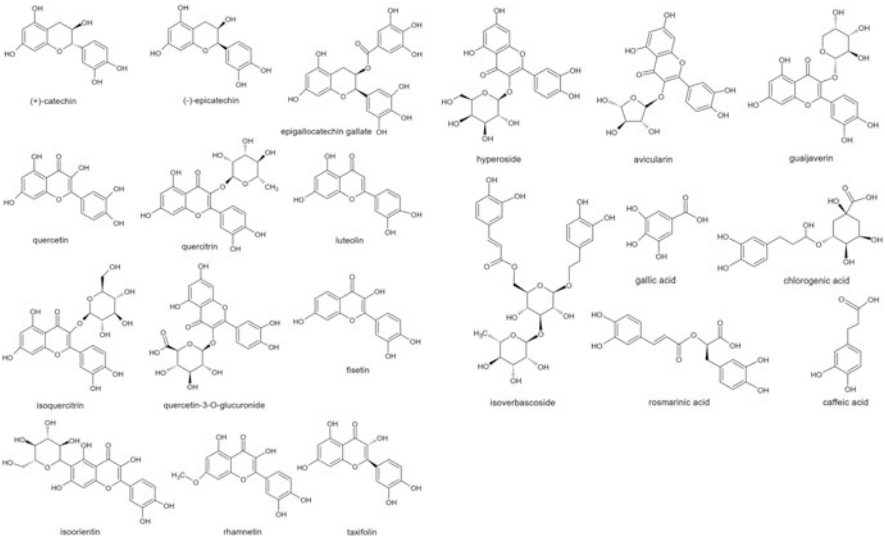



**Fig. 2** Panel of *Leishmania* arginase inhibitors. All the selected compounds displayed $IC_{50} < 10$ μM

site. In contrast, flavonoids that lack a catechol group such as apigenin, vitexin, and isovitexin, display no significant ARG inhibition [60, 62, 63].

Epigallocatechin gallate (EGCG), (+)-catechin, (−)-epicatechin, and gallic acid are polyphenols commonly found in green tea. These substances displayed LaARG inhibitory activity with $IC_{50}$ values of 3.8 ± 0.1, 0.77 ± 0.01, 1.8 ± 0.5, and 2.2 ± 0.1 μM, respectively. Interestingly, these polyphenols exhibited specificity toward *Leishmania* ARG, as the $IC_{50}$ for mammalian ARG was at least 250 times greater than that for parasite ARG [64]. Moreover, polyphenols proved to be active against *Leishmania infantum* ARG (LiARG). The catechol-containing caffeic acid and rosmarinic acid (a caffeic acid ester) inhibited 56.9 ± 5.5% and 71.4 ± 0.8%, respectively, of LiARG activity at 100 μM. In silico studies revealed that residues His140, Ala141, Asp142, Glu198, and Pro259, from the enzyme's active site, make direct contacts with rosmarinic acid through hydrogen bonds and π-stacking interactions. It is worth mentioning that these phenolic acids also exhibited inhibitory activity against *L. infantum* parasites [65]. Potent *Leishmania* arginase inhibitors are demonstrated in Fig. 2.

The *n*-butanolic fraction (BUF) obtained from the aqueous extract of *Stachytarpheta cayennensis* is a strong inhibitor of LaARG with an $IC_{50}$ of 1.2 μg/mL. Interestingly, the extract is less active against macrophage ARG ($IC_{50}$ = 1,000 μg/mL) and displays activity against intracellular amastigotes ($IC_{50}$ = 51 μg/mL). The antileishmanial activity of BUF was attributed to its major constituents, the caffeoyl phenylethanoid glycosides verbascoside and isoverbascoside [66]. In fact, verbascoside was capable of inhibiting LaARG with a Ki of 0.7 ± 0.1 μM. In addition, verbascoside prevented *L. amazonensis*

promastigote and amastigote growth with $IC_{50}$ values of 19 and 32 μM, respectively [67, 68].

The synthesis of new molecules is an interesting strategy for the development of potential enzyme inhibitors and further drug candidates. A series of [1,2,4]triazolo [1,5-a]pyrimidine scaffold derivatives containing $CF_3$ or $CH_3$ at the 2-position exhibited LaARG inhibition. Among them, the trifluoromethyl[1,2,4]triazolo [1,5-a]pyrimidine derivative presenting a $CF_3$ group in the 2-position, a $CH_3$ in the 5-position and a hydrazinecarbothioamide in the 7-position was the most active compound with an $IC_{50}$ of 16.5 ± 0.5 μM [69]. Fluorine is a wildly used substituent in the design of pharmacologically active molecules due to its contribution to hydrogen bonding, increase of lipophilicity and electrostatic interactions. Following the strategy of fluorine incorporation into a hydrazine scaffold, a series of α,-α-difluorohydrazides was synthetized as potential inhibitors of LaARG. Four compounds showed significant LaARG inhibition, including *N*-(2-(2-(2-carbamimidoylhydrazinyl)-1,1-difluoro-2-oxoethyl)phenyl)acetamide ($IC_{50}$ = 12 ± 2 μM), *N*-(2-(1,1-difluoro-2-oxo-2-(2-phenylhydrazinyl)ethyl)phenyl) acetamide ($IC_{50}$ = 12 ± 3 μM), *N*-(2-(2-(2-(4-(trifluoromethyl)phenyl)hydrazinyl)-1,1-difluoro-2-oxoethyl)phenyl)acetamid ($IC_{50}$ = 38 ± 2 μM), (2-acetamidophenyl)-*N*-benzyl-α,α-difluoroacetamide ($IC_{50}$ = 37 ± 6 μM). It is interesting to note that the phenylhydrazide moiety was fundamental for LaARG targeting and thus constitute a promising scaffold for the development of new antileishmanial agents [70].

*Leishmania* arginase displays structural differences from human arginase [71] and this difference may be exploited for the design of selective inhibitors. However, even inhibition of host's arginase may also characterize an advantage for the control of the disease. Indeed, L-arginine is a substrate for inducible nitric oxide synthase (iNOS) to produce citrulline and nitric oxide (NO). NO is the most important defense mechanism of macrophages against intracellular pathogens [72]. Previous reports suggested that NO triggers iron loss from enzyme(s) with iron–sulfur prosthetic groups, leading to enzymatic inhibition and parasite elimination [73]. Therefore, arginase inhibition may cause L-arginine accumulation in the cell, making it more available to iNOS [74–76].

The control of *Leishmania* infection is attributed to an efficient Th1 immune response, which includes the production of interleukin IL-12 and growth factor INF-γ, leading to macrophage activation and NO production. On the other hand, a Th2 immune response with a consequent production of anti-inflammatory interleukins (IL-10 and IL-4) and growth factor TGF-β contributes to disease progression [77]. This response also induces the synthesis of arginase by macrophages (Fig. 3). Previous reports have demonstrated that the increased expression of arginase by macrophages was proportional to the increase in parasite load and worsening of the disease [78, 79]. This phenomenon was observed on a murine model of infection with *Leishmania major* in which arginase activity led to L-arginine depletion at the site of lesion and affected the ability of local T cells to proliferate and produce IFN-γ [80]. It was clearly evidenced when BALB/c mice infected with *L. major* were treated with $N^{\omega}$-hydroxy-nor-L-arginine (nor-NOHA), a synthetic arginase

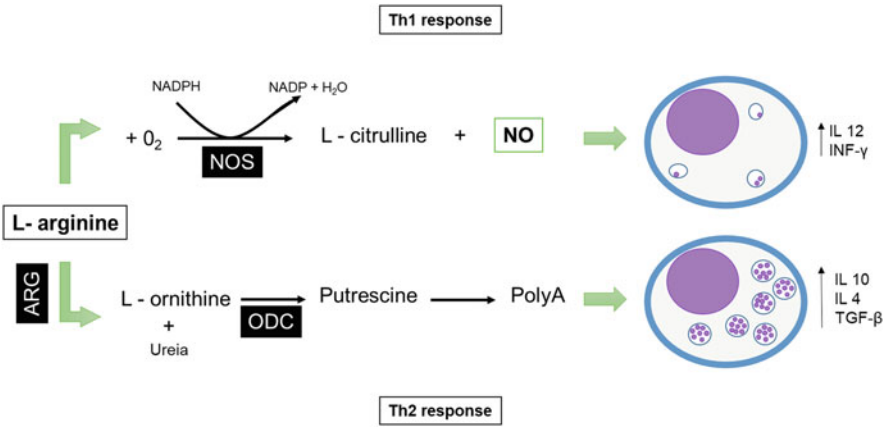



**Fig. 3** L-arginine metabolic pathways in macrophages. The course of infection by parasite is modulated by host immune response. A Th1 immune response leads to macrophage activation followed by NO production and the death of the parasites. On the other hand, the induction of arginase through a Th2 immune response may lead to parasite proliferation and establishment of infection

competitive inhibitor. An important reduction on both lesion size and parasite burden was observed for the treated group when compared to control [81]. The same effects were observed when human macrophages infected with *L. amazonensis* were treated with nor-NOHA. Indeed, the analysis of infected macrophage culture supernatants revealed a 50% decrease in TGF-β and the prostaglandin $E_2$ levels, both essential mediators of *Leishmania* infection, when compared to control. In addition, a proinflammatory response was observed due to the increase in TNF-α and IL-12 levels [82].

It is worth mentioning that $N^{\omega}$-hydroxyl-arginine (LOHA), a physiological arginase inhibitor, is capable of inhibiting arginase activity of *L. major* and *L. infantum* (~98%) at 100 μM. In addition, it reduces the intracellular amastigote burden on BALB/c mice macrophages infected with these parasites. Moreover, arginase activity of infected macrophages decreased from 25 ± 1.24 (untreated mice) to 1.23 ± 0.08 (LOHA-treated mice) without compromising of host cell viability. Taken together, these results suggest that the antiproliferative effect of LOHA may be related to both parasite and host cell arginase inhibition. In addition, a stable population of macrophages seems to be independent of polyamines for growth [83].

## 3.2 *Ornithine Decarboxylase (ODC)*

Ornithine decarboxylase (ODC) catalyzes the decarboxylation of ornithine to produce putrescine, a rate-limiting step in the biosynthesis of polyamines. A knockout strain of *Leishmania donovani* lacking the ODC gene is unable to grow and

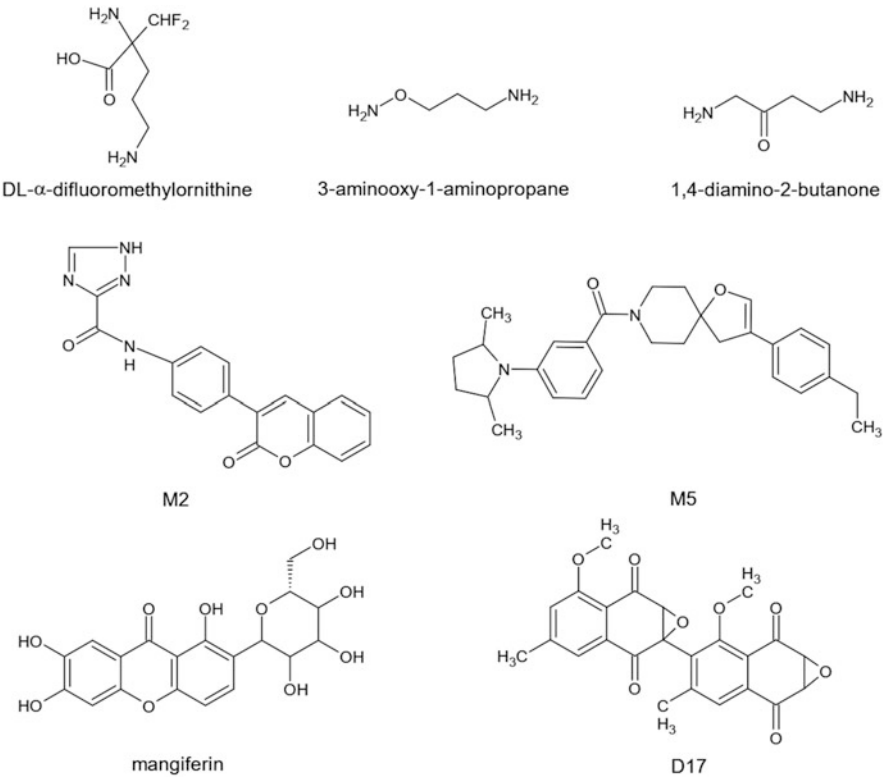



**Fig. 4** Panel of *Leishmania* ODC inhibitors

proliferate in the absence of putrescine, revealing that ODC is indispensable to parasite survival. Supplementation with spermidine, spermine, or other diamines, such as 1,3-diaminopropane or cadaverine, restored parasite growth, albeit with lower rates than putrescine [84]. The importance of ODC for leishmaniasis progression was evidenced by BALB/c mice infected with a *L. donovani* ODC mutant. Parasite load in the liver and spleen tissues were found to be three orders of magnitude lower than the control (mice infected with *L. donovani* wild type), suggesting that ODC activity is required to sustain a successful infection [85]. Below, some potent inhibitors of ODC are discussed. Moreover, chemical structures of ODC inhibitors are showed in Fig. 4.

DL-α-difluoromethylornithine (DFMO) is an irreversible inhibitor of ODC used to treat African sleeping sickness [86]. In addition to displaying cytotoxicity toward *Trypanosoma brucei*, DFMO showed inhibitory activity against *L. infantum* [87] and *L. donovani* [88] promastigotes ($IC_{50}$ of 38 and 30 μM, respectively). This growth inhibitory effect was reverted by exogenous putrescine, suggesting that DFMO mode of action is based on ODC inhibition. Structural investigation showed that DFMO binds at the active site of ODC, forming a Schiff base with PLP in one

monomer and a covalent bond with a Cys residue in the other monomer [89]. An in vivo study using *L. donovani*-infected golden hamster revealed that DFMO was more effective than the reference drug sodium antimony gluconate in the control of parasite burden on liver and spleen tissues, pointing at ODC as a major target for leishmaniasis chemotherapy [90]. Similar effects were observed for other fluorinated derivatives of L-ornithine, including Δ-MFMO and Δ-MFMOme [87].

3-aminooxy-1-aminopropane (APA), a putrescine analogue, was reported as an anti-*L. donovani* agent and its mechanism of action was attributed to ODC inhibition. The inhibitory effect of APA on ODC was evidenced by the reduction of the parasite levels of putrescine, spermidine, and trypanothione. In contrast to DFMO, APA binds at the substrate-binding site of ODC, next to PLP, but without the formation of an oxime [91]. APA displayed better results than DFMO in the inhibition of *L. donovani* promastigote and amastigote growth with $IC_{50}$ values of 42 μM and 5 μM, respectively. Interestingly, parasites overexpressing ODC exhibited lower sensitivity to sodium antimony gluconate and APA than wild type, suggesting a positive relationship between ODC and parasite resistance to drugs. Interestingly, *Leishmania* resistant strains from clinical isolates showed high levels of spermidine and putrescine, reinforcing the previous evidence that parasite resistance may be associated with an increase in ODC activity and, consequently, in the levels of spermidine and putrescine [92].

Using a structure-based drug screening approach, a library of 35,889 compounds were virtually screened against the homology model of *L. donovani* ODC, yielding 20 hits that showed preferred binding to *Leishmania* than human ODC. The top 20 compounds interacted with two conserved binding pockets at the enzyme, either the substrate binding or the catalytic site of ODC [93]. In a similar strategy, Grover et al. (2012) screened a dataset of 169,515 natural compounds against the three-dimensional models of *L. donovani* ODC and spermidine synthase (SpdS), another enzyme of the polyamine pathway, to find dual inhibitors of both enzymes. Out of these, two compounds, dihydrocitrinone (DHC) and (2R)-2-([1]benzofuro[3,2-d] pyrimidin-4-ylamino)-3-(1H-indol-3-yl)propanoate (BFPT), displayed high affinity binding to both ODC and SdpS, while failing to interact with human SpdS. These compounds were found to interact with the active site residues of ODC and SpdS via hydrogen bonds and hydrophobic interactions [94]. In a further study, a high throughput virtual screening of zinc database ligands revealed 12 compounds with good inhibition activity against ODC [95]. In the search for novel selective inhibitors of *L. donovani* ODC, the following compounds were identified: *N*-[4-(2-oxo-2H-chromen-3-yl)phenyl]-1H-1,2,4-triazole-3-carboxamide (M2), 8-[3-(2,5-dimethylpyrrol-1-l)benzoyl]-3-(4-methoxyphenyl)-1-oxa-8-azaspiro[4.5] dec-2-ene (M5), and 1,3,6,7-tetrahydroxyxanthone C2-b-D-glucoside (mangiferin). Among them, M5 showed better results, displaying the lowest $IC_{50}$ value (125 μM) against *L. donovani* promastigotes, regardless of the high $K_i$ (370.63 μM) against the enzyme [96].

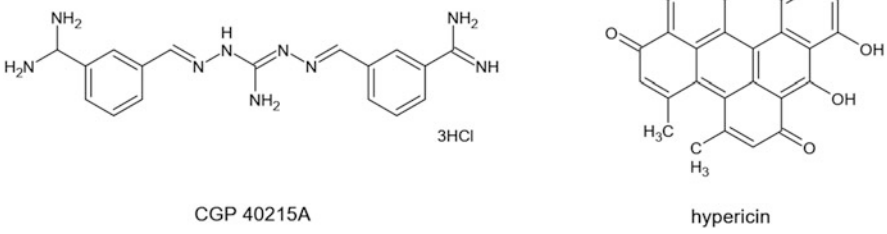
The diospyrin derivative diepoxide naphthoquinonoid (D17) was reported as a non-competitive inhibitor of *L. donovani* ODC. The docking analysis showed hydrogen bond interactions between the compound and the enzyme's active site

residues Lys135 and Arg120. Moreover, D17 was able to eliminate *L. donovani* promastigotes and intracellular amastigotes at 7.2 ± 1.8 and 0.18 ± 0.005 μM ($IC_{50}$ values), respectively. Despite its moderate toxicity, D17 is certainly a promising drug candidate for visceral leishmaniasis treatment [97].

## 3.3 S-Adenosylmethionine Decarboxylase (AdoMetDC)

AdoMetDC catalyzes the decarboxylation of S-adenosylmethionine (AdoMet) to produce S-adenosyl-5′-3-methylthiopropylamine (dcAdoMet), which is responsible for donating the aminopropyl group to the biosynthesis of spermidine. A *L. donovani* knockout strain carrying an AdoMetDC gene deletion was unable to proliferate in the absence of high concentrations of spermidine, suggesting that AdoMetDC is essential to parasite survival and validating this enzyme as a potential therapeutic target [98]. AdoMetDC has been shown to physically interact with spermidine synthase (SpdS) so that the product of the first enzyme is directly channeled to the next enzyme's active site. The AdoMetDC-SpdS heteromeric complex seems to be structurally different than its human counterpart, providing a rationale for the dual inhibition of both enzymes as a potential therapeutic strategy [99].

The polyamine analogue CGP40215A (Fig. 5), a specific inhibitor of *Leishmania* AdoMetDC, displayed inhibitory activity against *L. donovani* promastigotes with an $IC_{50}$ value of 18 μM. The antileishmanial effect was reversed by the supplementation of spermidine and spermine, suggesting that the main cellular target of CGP40215A is, in fact, AdoMetDC. CGP40215A showed a synergistic effect with other inhibitors of the polyamine pathway, including DFMO and MDL 27695 [100]. In addition to CGP40215A, other polyamine analogs showed leishmanicidal activity [101]. Moreover, the antileishmanial activity of Berenil and Methylglyoxal bis (guanylhydrazone) (MGBG) was associated with AdoMetDC inhibition [102].




CGP 40215A hypericin

**Fig. 5** *Leishmania* AdoMetDC (left) and SpdS (right) inhibitors

### 3.4 Spermidine Synthase (SpdS)

SpdS catalyzes the conjugation of the aminopropyl group from decarboxylated AdoMet to putrescine, producing spermidine. A *L. donovani* strain deficient in SpdS was unable to proliferate in the absence of polyamines and required spermidine for sustained growth. In addition, the SpdS null mutant had its ability to infect BALB/C mice severely compromised with a reduction of three orders of magnitude in parasitic load of liver and spleen when compared to wild type, indicating that SpdS is essential for parasite viability and infectivity and pointing at this enzyme as a valid therapeutic target [103].

Despite its relevance, few studies have identified specific inhibitors of *Leishmania* SpdS. Currently, there is no experimentally derived three-dimensional structure of *Leishmania* SpdS. Thus, a homology model of *L. donovani* Spds was constructed based on the crystal structure of *T. cruzi* SpdS. A dataset of one million compounds was virtually screened against this homology model. Two compounds were selected as specific SpdS inhibitors, based on their binding affinity and selectivity toward parasite SdpS in contrast to the human enzyme [104]. In addition, dual inhibitors of natural origin of ODC and SpdS were reported, namely DHC and BFPT, which were described in the previous section. Moreover, the natural compound fallacinol displayed stronger binding affinity toward Spds than ODC, representing a specific SpdS inhibitor [96]. Using an in silico approach, two terpenoids, geraniol and linalool, which are structurally homologous to the substrate putrescine, were found to bind avidly to *L. donovani* Spds with binding free energies ($\Delta G^{\text{bind}}$) of −43 and −73 kJ/mol, respectively [105]. Hypericin (Fig. 5), a natural compound from *Hypericum perforatum*, is the only experimentally validated SpdS inhibitor described to date. Hypericin binds at the active site of *L. donovani* SpdS, while stabilizing its gatekeeping loop. Hypericin inhibited recombinant SpdS with a $K_i$ of 3.68 μM, with respect to dcAdoMet, and displayed inhibitory activity against *L. donovani* promastigotes with an $IC_{50}$ of 18 μM. Supplementation with spermidine restored parasite viability, suggesting that hypericin activity is due to spermidine starvation and specific SpdS inhibition [106].

## 4 Trypanothione Pathway

Cells generate reactive oxygen species (ROS) and reactive nitrogen species (RNS) as part of their metabolic function. These molecules participate in various cellular functions, such as cell division, differentiation, and metabolism. However, the excess of ROS and RNS induces damage to biological macromolecules, including lipids, proteins, and DNA, impairing cellular function or even leading to cell death. Thus, a functional antioxidant defense is critical for the proper control of ROS and RNS levels [107, 108].

Most aerobic organisms possess an antioxidant system based on two fundamental molecules, glutathione and thioredoxin. The tripeptide glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) is capable of scavenging ROS and RNS, protecting cells from oxidative damage. It constitutes the major redox buffer in most cells as it receives one electron from ROS to generate the oxidized glutathione dimer (GSSG). Thioredoxin, on the other hand, is a key antioxidant enzyme that uses its protein disulfide reductase activity as a defense mechanism. It provides electrons for thiol-dependent peroxidases that directly remove ROS and RNS. Together, these two antioxidant systems are responsible for maintaining cellular redox homeostasis [109, 110].

Trypanosomatids are able to synthesize glutathione and thioredoxin; however, they lack the genes encoding glutathione reductase and thioredoxin reductase, enzymes essential for recycling reduced glutathione and thioredoxin, respectively. In contrast, trypanosomatids exhibit an antioxidant system based on a unique molecule named trypanothione [111]. Trypanothione is composed of two molecules of glutathione bound to one molecule of spermidine. Thus, spermidine constitutes a link between the polyamine and trypanothione pathways (Fig. 1). This system is similar to the glutathione system: trypanothione shows direct antioxidant activity; there are antioxidant enzymes whose activity depends on trypanothione; trypanothione is recycled by a reductase enzyme that reduces it again [112].

Previous studies have shown that several antileishmanial agents induce ROS production and that addition of the antioxidant NAC prevents parasite death [113]. Increased expression of trypanothione-related enzymes has been correlated to drug resistance in certain parasite strains [114]. In addition, it has been shown that trypanothione binds to some drugs, such as pentavalent antimonials, inducing their excretion [115]. Thus, due to the lack of the trypanothione system in mammals, the non-redundancy of the thiol redox system in parasites, and the sensitivity of *Leishmania* to oxidative stress, the enzymes involved in the trypanothione pathway are regarded as therapeutic targets for antileishmanial drugs. In the next sessions, we describe each of the enzymes that compose the trypanothione pathway in *Leishmania* together with their respective inhibitors reported so far.

## *4.1 Glutathionylspermidine Synthetase (GspS)*

Biosynthesis of glutathionylspermidine (GspdSH) constitutes the first step of trypanothione pathway. GspS is one of the two enzymes able to catalyze the conjugation of spermidine to glutathione. Genome analysis showed that GspS is not expressed in all trypanosomatids, including *Leishmania* species. Indeed, *L. infantum* and *L. mexicana* retains GspS full length gene, while a pseudogene was observed in *L. major* and *L. braziliensis*. Among the *Leishmania* species, *L. donovani* and *L. amazonensis* lack the GspS gene [116, 117]. Interestingly, despite being expressed by *L. infantum*, GspS was reported as not essential for parasite survival. It was demonstrated that a $GspS^{-/-}$ line was able to replicate in

both evolutive stages, promastigote and amastigote [118]. Therefore, GspS has been disqualified as a therapeutic target for leishmaniasis treatment.

### 4.2 Trypanothione Synthetase (TryS)

TryS catalyzes the biosynthesis of trypanothione by the consecutive conjugation of two glutathione molecules to spermidine. During the catalytic cycle, glutathionylspermidine serves as the reaction intermediate, which happens at the expense of two ATP molecules [119, 120]. A *L. infantum* knockout strain in which both TryS alleles were mutated is not viable. In addition, complementation with an episomal copy of the gene demonstrated the essentiality of TryS for *L. infantum* promastigote and amastigote survival [118]. As TryS is specific to *Leishmania*, with no human ortholog, and there is no bypass to the biosynthesis of trypanothione, TryS is considered a highly druggable enzyme and a valuable therapeutic target [121].

TryS is a bifunctional enzyme that catalyzes both the biosynthesis and the hydrolysis of trypanothione. The crystal structure of *L. major* TryS revealed that each activity resides in a separate domain of the enzyme [120]. Moreover, the experimental three-dimensional structure enabled the virtual screening of potential TryS inhibitors. An in silico study docked 123 sesquiterpene derivatives with proven antiparasitic activity against the crystal structure of *L. major* TryS, revealing two sesquiterpene coumarins as selective enzyme inhibitors [122]. Using the homology model of *L. infantum* TryS, Khademvatan et al. (2019) showed that polyphenolic compounds commonly found in green tea, such as catechin, (−)-epicatechin, epicatechin gallate (ECG), and (−)-epigallocatechin3-*O*-gallate (EGCG), bind to key residues in the active site of the enzyme. Among them, EGCG proved to be the best inhibitor, displaying the greatest binding affinity ($\Delta G^{\text{bind}} = -8.49$ kcal/mol) and inhibitory activity against *L. infantum* promastigotes ($IC_{50} = 27.71$ μM) [123]. In a similar approach, Mehwish et al. (2019) identified the naturally occurring flavonoid rutin as a ligand of TryS's active site, while exhibiting inhibitory effect against promastigote and amastigote forms of *L. tropica* with $IC_{50}$ values of 91.2 and 101.3 μg/mL, respectively [124]. Finally, docking studies revealed effective interactions of glyburide, a drug used in the treatment of diabetes, with the active site residues of *L. donovani* TryS, supporting the repurposing of this drug as a potential antileishmanial agent [125].

In addition to the theoretical work, a few other studies revealed experimental inhibitors of TryS. The natural compounds tomatine, conessine, uvaol, and betulin were shown to inhibit recombinant *L. donovani* TryS ($K_i$ of 12.5, 3.12, 3.55, and 6.33 μM, respectively), while exhibiting inhibitory activity against parasite promastigotes ($IC_{50}$ of 18.02, 13.42, 11.23, and 11.71 μM, respectively) [126]. Due to their ability to bind to the ATP-binding pocket of TryS, paullones (7,12-dihydroindolo[3,2-d][1]benzazepin-6(5 H)-ones) were found as promising inhibitors of this enzyme from various parasite species [127]. A paullone derivative substituted at the lactam nitrogen $N^5$, known as FS-554, was capable of inhibiting

recombinant TryS from *L. infantum* at the nanomolar range ($IC_{50}$ of 349.57 ± 47.4 nM), albeit displaying a slightly poorer effect against parasite promastigotes ($IC_{50}$ of 112.3 ± 1.1 μM) [118]. Benítez et al. (2016) developed a high throughput assay that enabled the screening of a 144-compound library, containing 7 different chemical families, against recombinant purified *L. infantum* TryS. The search identified two $N^5$-substituted paullone derivatives as nanomolar-affinity inhibitors, namely FS-554, which was described previously, and MOL2008. MOL2008 was the most potent enzyme inhibitor with an $IC_{50}$ of 150.0 ± 6.0 nM. Regarding biological activity, MOL2008 ($IC_{50}$ = 12.6 ± 1.6 μM) exhibited a ten-fold increase in inhibitory activity against *L. infantum* promastigotes when compared to FS-554 ($IC_{50}$ = 112.3 ± 1.1 μM), pointing at amide-substituted paullones as promising chemical scaffolds. Moreover, MOL2008 depleted the intracellular levels of trypanothione, suggesting specific TryS inhibition and on-target effect [128]. Finally, Saudagar et al. (2013) revealed oxabicyclo[3.3.1] nonanones as inhibitors of TryS from *L. donovani* ($K_i$ = 14.2 ± 0.8 μM). These compounds also inhibited *L. donovani* promastigote growth with an $IC_{50}$ of 4.9 ± 0.4 μM, leading to an increase in intracellular ROS, trypanothione depletion, mitochondrial damage, and apoptosis [129]. Other inhibitors of this enzyme include glutathione derivatives [130] (Fig. 6).

### 4.3 Trypanothione Reductase (TryR)

TryR catalyzes the reduction of trypanothione disulfide into trypanothione dithiol in a NADPH-dependent manner. Reduced trypanothione plays a central role during *Leishmania* infection due to its antioxidant capacity. It provides reducing equivalents for tryparedoxin/tryparedoxin peroxidase, which in turn detoxify hydrogen peroxide produced by infected macrophages [113]. *L. donovani* and *L. major* strains with downregulated TryR expression showed attenuated infectivity and less capacity to survive inside activated murine macrophages [131, 132]. The difficulty in obtaining viable TryR null mutants provides additional evidence for the essentiality of this enzyme. In addition to being essential, TryR is a trypanosomatid-specific enzyme. It shares similarities with glutathione reductase, its closest human ortholog; however, structural differences in the active site of the two enzymes provide a rationale for selective inhibition, contributing to the high druggability of TryR [133].

The crystal structure of TryR from *L. infantum* revealed a homodimer formed by three functionally different domains, the NADPH-binding domain, the FAD-binding domain, and the substrate-binding or interface domain. During the reaction mechanism, electrons flow from NADPH to the flavin nucleus to a cysteine disulfide at the active site. Then, reduced Cys52 forms a mixed disulfide with trypanothione, which is attacked by Cys57, generating reduced trypanothione and the oxidized enzyme [11, 24]. Antimonials, which are front-line drugs in the treatment of leishmaniasis, have been shown to interfere with trypanothione metabolism and inhibit TryR [23, 24]. The molecular basis of inhibition involves coordination of the trivalent

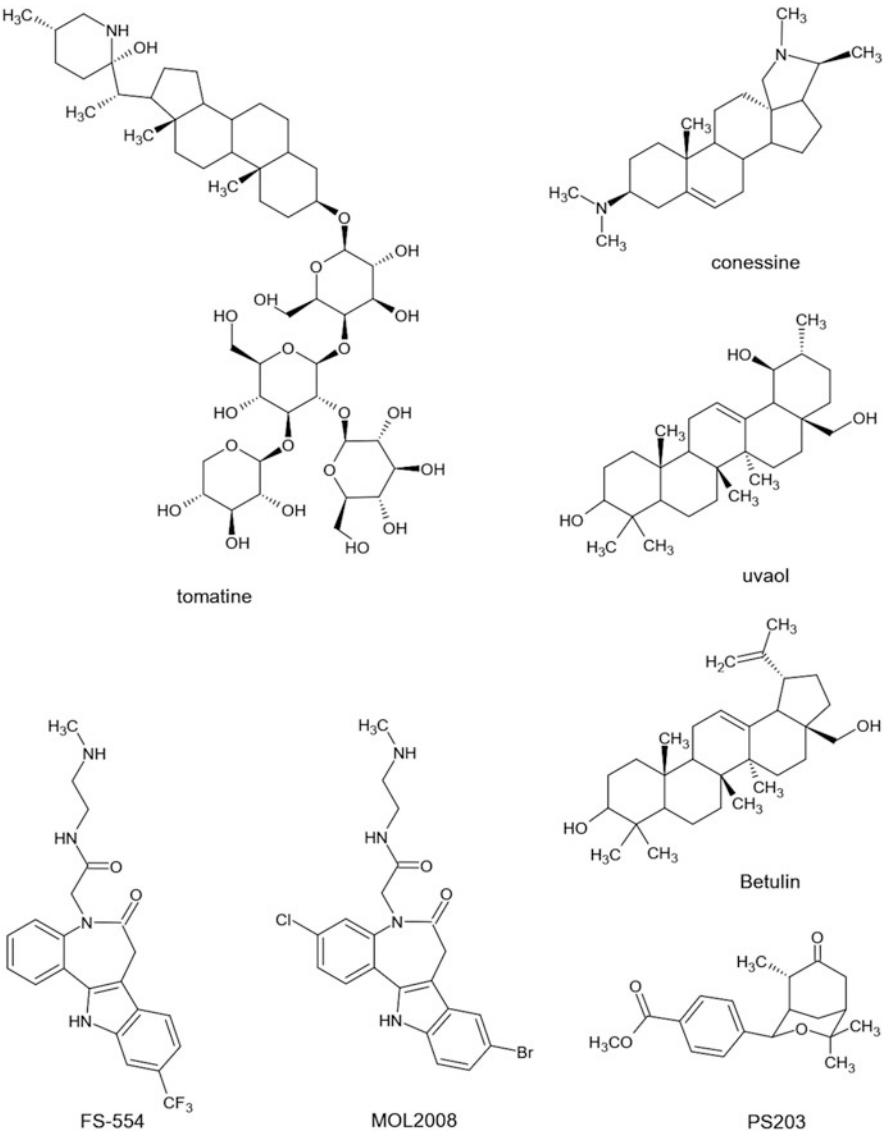



**Fig. 6** Panel of *Leishmania* TryS inhibitors

antimony ion by active site residues, including the two catalytic cysteines (Cys 52 and Cys 57) as well as Thr335 and His461. This further validates TryR inhibition as a bona fide therapeutic strategy. As TryR is considered a valuable drug target, the current literature on the inhibition of this enzyme is quite vast. Thus, for simplicity, we will focus only on those compounds that were shown to inhibit TryR

experimentally; compounds that only had evidence of interaction in silico will not be addressed.

In addition to antimonials, other metals and metal-based compounds have been shown to inhibit TryR activity. Auranofin, a gold complex largely used as an antiarthritic drug, is a potent inhibitor of *L. infantum* TryR ($K_i = 155 \pm 35$ nM), thereby displaying antileishmanial effect against promastigotes ($IC_{50}$ of $9.68 \pm 1.2$ μM) [134]. Collotti et al. (2013) investigated the inhibitory activity of a set of structurally different gold-containing compounds on *L. infantum* TryR. Among them, $(Cl_2Au(III)(Pbi)Au(I)(PPh_3))(PF_6)$ showed the best results with a $K_i$ value of $22 \pm 11$ nM [135]. Silver nanoparticles encapsulated with ferritin molecules were shown to effectively inhibit *L. infantum* TryR ($K_i$ of $500 \pm 200$ nM and $50 \pm 10$ nM for Ag(0) and Ag(I), respectively), while exhibiting inhibitory activity against parasite promastigotes ($IC_{50}$ of $2.18 \pm 0.33$ μM) and intracellular amastigotes ($IC_{50}$ of $1.76 \pm 0.24$ μM). The mechanism of inhibition of gold and silver compounds proved to be similar to that observed for antimonials and involves binding to the catalytic cysteine residues [136]. Other metal-containing compounds inhibited trypanosomatids, including *Leishmania* [137].

Azol-based compounds have been identified as inhibitors of *Leishmania* TryR. Baiocco et al. (2013) showed that a diarylpyrrole, namely 4-((1-(4-ethylphenyl)-2-methyl-5-(4-(methylthio)phenyl)-1H-pyrrol-3-yl)methyl)thio-morpholine, was active against *L. infantum* TryR ($K_i$ of $4.6 \pm 2.5$ μM) and inhibited intracellular amastigote growth ($IC_{50}$ of 13.77 μM), validating this chemical scaffold. Structural characterization of the enzyme-inhibitor complex revealed that the compound binds at the substrate-binding site of TryR, competing with trypanothione [138]. Chemical structure of diarylpyrrole and other TryR inhibitors discussed below are showed in Fig. 7.

Baquedano et al. (2016) reported the antileishmanial activity of a series of selenocyanates and diselenides carrying various chemical skeletons (quinoline, quinoxaline, acridine, furante, isosazole, etc). A top hit, namely 3,5-dimethyl-4-isoxazolyl selenocyanate, was selected based on its effectiveness in inhibiting recombinant TryR activity ($IC_{50}$ of $0.46 \pm 0.01$ μM) as well as *L. infantum* axenic ($IC_{50}$ of $0.73 \pm 0.10$ μM) and intracellular ($IC_{50}$ of $23.2 \pm 4.3$ μM) amastigotes, pointing at this compound as a promising drug candidate [139].

Diaryl sulfides proved to be potent inhibitors of *Leishmania* TryR. Saccoliti et al. [140] evaluated the antileishmanial activity of a set of diaryl sulfide derivatives against *L. infantum* promastigotes. Among them, compound RDS777, (6-(sec-butoxy)-2-((3-chlorophenyl)thio)pyrimidin-4-amine), showed the best biological activity ($IC_{50} = 29.43$ μM), while potently inhibiting parasite TryR ($K_i = 0.25 \pm 0.18$ μM). The crystal structure of RDS777-bound TryR revealed that the compound binds at the active site of the enzyme, making hydrogen bonds with catalytic residues, including Cys52, Cys57, and Glu466 [140]. In an attempt to find better, more selective TryR inhibitors, Colotti et al. (2020) designed a series of diaryl sulfide analogues based on the three-dimensional structure of the TryR-RDS777 complex. Promising results were obtained with compound RDS562, 2-chloro-6-(phenylthio)pyrimidin-4-amine, which displayed activity against

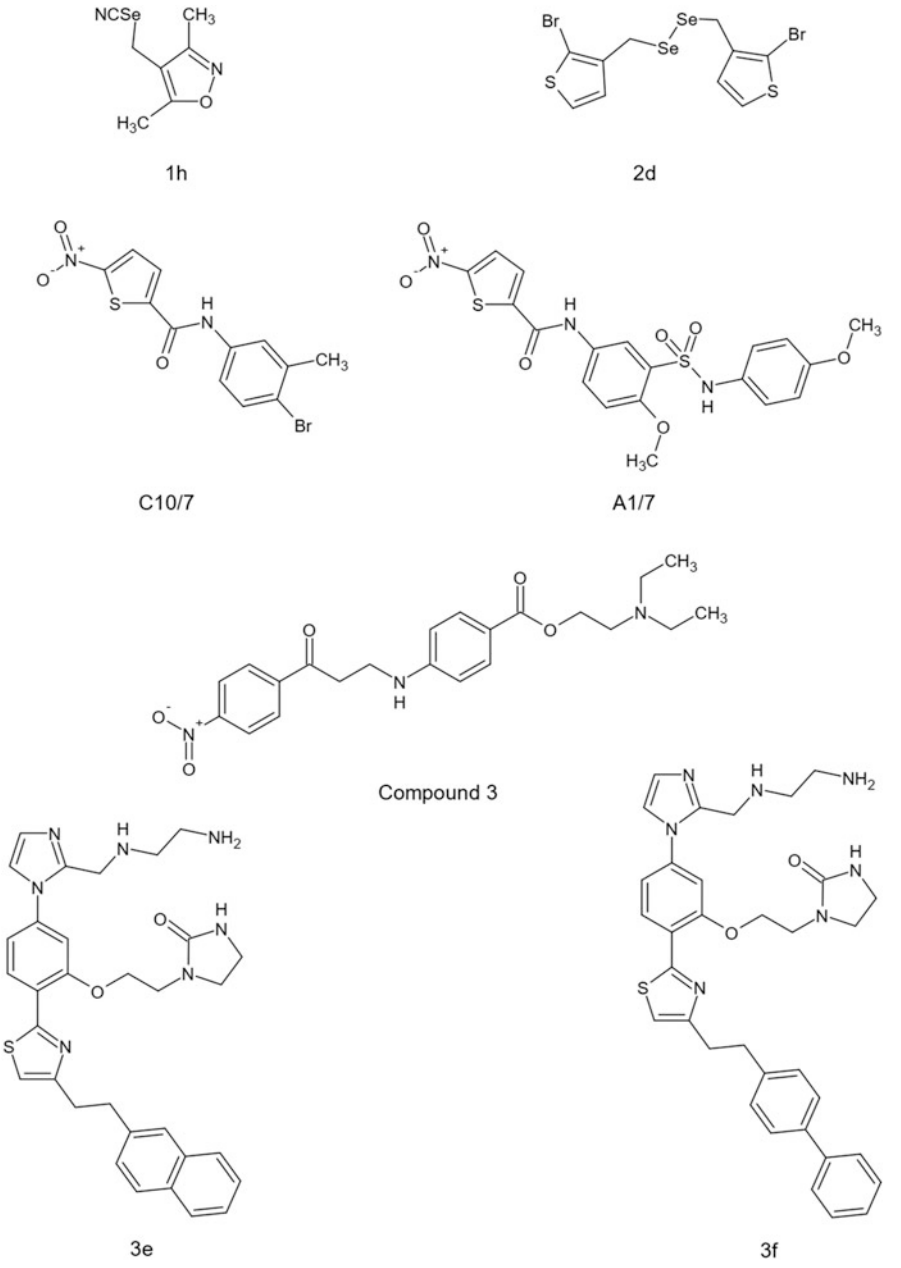



**Fig. 7** Panel of *Leishmania* TryR inhibitors

*L. infantum* promastigotes at the micromolar range ($IC_{50}$ = 11.0 ± 2.0 μM) and competitively inhibited recombinant purified TryR ($K_i$ = 12.0 ± 1.0 μM), while decreasing the intracellular levels of trypanothione by 30%. RDS562 was unable to inhibit human glutathione reductase and thus exhibited increased selectivity toward parasite TryR. Interestingly, the mechanism of action of RDS562 differed from that of RDS577, in which the compound interacted with residues from the trypanothione-binding site instead of the active site of TryR [141].

Chalcone derivatives have been shown to exhibit antileishmanial effect through TryR inhibition. From a set of 31 substituted chalcones, Ortalli et al. (2018) identified a hit compound, (E)-1-(2-methoxy-4-((3-methylbut-2-en-1-yl)oxy)phenyl)-3-(4-nitrophenyl)prop-2-en-1-one, which displayed an $IC_{50}$ value of 3.0 μM against promastigotes and 14 μM against intracellular amastigotes of *L. donovani*, while showing low toxicity to mammalian cells (SI of 200). In addition, this compound inhibited parasite TryR at nanomolar concentration ($K_i = 0.45 \pm 0.11$ μM). Docking studies suggested that the chalcone analogue interacts with the trypanothione-binding site, lying at a hydrophobic pocket close to the catalytic cysteines [142].

Ilari et al. (2018) screened the 192 best antileishmanial compounds in the GlaxoSmithKline Leishbox, designed from a 1.8 million-compound library, against *L. infantum* TryR and identified three highly potent and selective inhibitors. The best hit, *N*-(4-bromo-3-methylphenyl)-5-nitrothiophene-2-carboxamide, displayed effective inhibition of *L. infantum* TryR activity ($IC_{50}$ = 190 nM). Docking analysis revealed the molecular basis of inhibition, indicating that the hit compound binds at the active site of the enzyme [143]. Using a high throughput approach, Turcano et al. (2018) developed and validated a luminescent assay that enabled the screening of a 120,000-compound library, leading to the discovery of a new class of TryR inhibitors. A hit compound, namely 2-(diethylamino)ethyl4-((3-(4-nitrophenyl)-3-oxopropyl)amino)benzoate, displayed activity against recombinant purified TryR ($IC_{50}$ of 7.5 ± 2.5 μM) and selectivity with respect to human glutathione reductase, while inhibiting *L. infantum* promastigote growth ($IC_{50}$ of 12.44 ± 1.09 μM). The crystal structure of the enzyme-inhibitor complex revealed that the compound binds at a unique site at the NADPH cavity entrance, providing further evidence for the druggability of TryR [144].

Recently, Revuelto et al. (2019) developed an interesting and innovative strategy to inhibit *L. infantum* TryR based on the disruption of its dimerization interface. Previously, linear and cyclic peptides, derived from an α-helix located at the dimerization interface of the enzyme, were shown to dissociate and inhibit TryR activity thereby displaying antileishmanial effects [145]. However, to increase their potency toward *L. infantum* parasites, conjugation with cell-penetrating peptides was required [146, 147]. To design small molecules that retained the TryR disrupting capacity of the peptide inhibitor, the α-helical mimetics pyrrolopyrimidine and 5-6-5-imidazole-phenyl-thiazole were used as scaffolds. Fifteen derivatives were synthesized and screened according to their ability to dissociate/inhibit *L. infantum* TryR and their biological activity. The naphthyl and biphenyl analogues of the imidazole-phenyl-thiazole series were the most potent inhibitors of TryR ($IC_{50}$ of

5.1 ± 0.4 and 8.6 ± 1.4 μM, respectively). In addition, they exhibited inhibitory effect against *L. infantum* promastigote ($IC_{50}$ of 12.8 ± 0.7 and 5.3 ± 0.3 μM, respectively) and intracellular amastigote forms ($IC_{50}$ of 12.8 ± 1.3 and 5.3 ± 0.2 μM, respectively), evidencing imidazole-phenyl-thiazole with bulky substituents as promising drug candidates [148].

Table 1 summarizes the antileishmanial agents that experimentally inhibit the enzymes addressed in this review.

## 5 General Considerations

This chapter summarizes the current literature on the inhibition of the polyamine and trypanothione pathways in *Leishmania*. Over the years, enzymes of these two pathways have proven to be good targets for the development of new antileishmanial drugs. Based on our review, we infer that arginase and trypanothione reductase are the two most studied enzymes, for which the largest number of inhibitors have been prospected. Many of the inhibitors presented here represent good hits that may be further optimized by medicinal chemistry studies. Moreover, further structural studies are needed to deepen our current understanding of the various mechanisms of inhibition, enabling the rational design of better inhibitors. In conclusion, we suggest that the studies presented here pave the way for the discovery of more effective, selective, and less toxic antileishmanial agents.

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**Compliance with Ethical Standards** *Conflict of Interest*: Author IAR declares that he has no conflict of interest. Author ARG declares that she has no conflict of interest. Author MMP declares that she has no conflict of interest. Author RGDGJ declares that he has no conflict of interest. Author ACFA declares that she has no conflict of interest. Author ASP declares that he has no conflict of interest.

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*Ethical Approval*: This article does not contain any studies with human participants or animals performed by any of the authors.

*Informed Consent*: All authors have given their consent to this book chapter.

**Table 1** Prospected compounds with inhibitory activity against enzymes belonging to *Leishmania* polyamine and trypanothione pathways

| Inhibitor | Source | Target enzyme | Percentage of inhibition (EC μM) | $K_i$ (μM) | Type of inhibition | *Leishmania* species | Parasite $IC_{50}$ μM (evolutive form) | Reference |
|---|---|---|---|---|---|---|---|---|
| Orientin | Natural/ *C. pachystachya* | rARG | 50 (15.9) | n.d. | n.d. | *L. amazonensis* | n.d. | [60] |
| | Natural | | 50 (16 ± 2) | | Uncompetitive | | | [63] |
| Isovitexin | Natural/ *C. pachystachya* | | 14 ± 3 (20) | | n.d. | | | [60] |
| Chlorogenic acid | Natural/ *C. pachystachya* | | 67 ± 5 (20) | | | | | |
| | *Natural* | | 50 (8.3 ± 0.2) | 5 ± 1 | Competitive | | > 500 (pro) | [149] |
| | | | 36.8 ± 1.9 (100) | n.d. | n.d. | *L. infantum* | n.d. | [65] |
| (+)-Catechin | Natural/ *C. pachystachya* | | 66 ± 8 (20) | | | *L. amazonensis* | | [60] |
| | *Natural/green tea* | | 50 (0.77 ± 0.001) | 12 ± 2.5 | Competitive | | | [64] |
| | *Natural* | | 49.02 ± 14.9 (100) | n.d. | n.d. | *L. infantum* | 395 ± 50 (pro) 286.9 ± 36.5 (ama) | [65] |
| (−)-Epicatechin | Natural/ *C. pachystachya* | | 62 ± 7 (20) | | | *L. amazonensis* | n.d. | [60] |
| | Natural/*green tea* | | 50 (1.8 ± 0.5) | 3 ± 0.4 | Competitive | | | [64] |
| Isoquercitrin | Natural/ *C. pachystachya* | | 54 ± 6 (20) | n.d. | n.d. | | | [60] |
| | | | 50 (3.8 ± 0.04) | 6.9 ± 0.3 | Non-competitive | | | [62] |
| Quercetin | Natural | | 50 (4.3 ± 0.03) | 8 ± 1 | Mixed | | | |
| | | | 67.05 ± 10.3 (100) | n.d. | n.d. | *L. infantum* | | [65] |
| | | | 50 (4.9 ± 0.5) | 5 ± 1 | Mixed | *L. amazonensis* | | [61] |

(continued)

**Table 1** (continued)

| Inhibitor | Source | Target enzyme | Percentage of inhibition (EC μM) | $K_i$ (μM) | Type of inhibition | *Leishmania* species | Parasite $IC_{50}$ μM (evolutive form) | Reference |
|---|---|---|---|---|---|---|---|---|
| Quercitrin | | | 50 (10 ± 0.08) | 7.2 ± 0.9 | Non-competitive | | | [62] |
| Fisetin | | | 50 (1.49 ± 0.3) | 1.9 ± 0.5 | Mixed | | | [63] |
| | | ARG (cell extract) | <5[a] (0.283) | n.d. | n.d. | *L. infantum* | 0.283 (pro)<br>0.102 (ama) | [150] |
| Luteolin | | rARG | 50 (9 ± 1) | 8 ± 1 | Mixed | *L. amazonensis* | n.d. | [63] |
| 7,8-Dihydroxyflavone (**DHF**) | | | 50 (12 ± 1) | 7.4 ± 0.4 | Non-competitive | | | |
| Isoorientin | | | 50 (9 ± 1) | n.d. | Uncompetitive | | | |
| Kaempferol | | | 50 (~50) | | n.d. | | | |
| Galangin | | | 50 (~100) | | | | | |
| Epigallocatechin gallate (**EGCG**) | *Natural/green tea* | | 50 (3.8 ± 0.1) | 4 ± 0.5 | Mixed | | | [64] |
| Galic acid | | | 50 (2.2 ± 0.2) | 7.2 ± 1.4 | Non-competitive | | | |
| Di-hydroquercetin | Natural | | 23.8 ± 6.95 (100) | n.d. | n.d. | *L. infantum* | | [65] |
| Caffeic acid | | | 56.9 ± 5.5 (100) | | | | 60.8 ± 11 (pro)<br>21.9 ± 5.0 (ama) | |
| | | | 50 (1.5 ± 0.3) | 0.5 ± 0.1 | Competitive | *L. amazonensis* | > 500 (pro) | [149] |
| Rosmarinic acid | | | 71.4 ± 0.8 (100) | n.d. | n.d. | *L. infantum* | 57.3 ± 2.65 (pro)<br>7.9 ± 1.7 (ama) | [65] |
| | | | 50 (2.1 ± 0.3) | 1.8 ± 0.3 | Competitive | *L. amazonensis* | 61 (pro) | [149] |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Apigenin-7-*O*-glycoside | | | 33.5 ± 2.04 (100) | n.d. | n.d. | *L. infantum* | n.d. | [65] |
| Iso-rhamnetin | | | 56.3 ± 5.6 (100) | | | | 818 ± 30 (pro) | |
| Rhamnetin | | | 56.7 ± 22.2 (100) | | | | 832 ± 6.9 (pro) | |
| | | | 50 (5.5 ± 0.4) | 4.3 ± 1.7 | Mixed | *L. amazonensis* | n.d. | [61] |
| Raponticin | | | 53.9 ± 5.1 (100) | n.d. | n.d. | *L. infantum* | >1,000 (pro) | [65] |
| Eugenol | | | 19.1 ± 2.05 (100) | | | | n.d. | |
| Verbascoside | | | n.d. | 0.7 ± 0.1 | Competitive | *L. amazonensis* | 19 (pro) | [67] |
| | | ARG (cell extract) | 100 (50) | n.d. | n.d. | | 32 (ama) | [68] |
| Isoverbascoside | | rARG | 50 (2.3 ± 0.3) | 1.0 ± 0.1 | Non-competitive | | n.a. (pro) | [149] |
| Cryptochlorogenic acid | | | 50 (11 ± 1) | 12.3 ± 0.1 | | | n.d. | |
| Rutin | | | 50 (10.4 ± 0.8) | 12.8 ± 0.4 | Mixed | | | [61] |
| Avicularin | | | 50 (5.6 ± 0.5) | 4.8 ± 0.9 | Competitive | | | |
| Guaijaverin | | | 50 (6.9 ± 0.2) | 4.4 ± 0.8 | | | | |
| Hyperoside | | | 50 (5.1 ± 0.5) | 2.7 ± 0.5 | | | | |
| Quercetin-3-*O*-glucuronide | | | 50 (8.2 ± 0.4) | 4.7 ± 0.4 | | | | |
| Taxifolin | | | 50 (1.6 ± 0.1) | 0.9 ± 0.2 | | | | |
| 2-(5-Methyl-2-(trifluoromethyl)-[1,2,4]triazolo [1,5-a]pyrimidin-7-yl) hydrazinecarbothioamide | Synthetic | | 50 (16.5 ± 0.5) | 17 ± 1 | Non-competitive | | | [69] |
| *N*-(2-(2-(2-Carbamimidoylhydrazinyl)-1,1-difluoro-2-oxoethyl)phenyl)acetamide | | | 50 (12 ± 2) | 5.1 ± 1.4 | Competitive | | 12.7 ± 0.3 (pro) | [70] |
| *N*-(2-(1,1-Difluoro-2-oxo-2-(2-phenylhydrazinyl)ethyl)phenyl)acetamide | | | 50 (12 ± 3) | 1.3 ± 0.8 | Mixed | | > 100 (pro) | |
| *N*-(2-(2-(2-(4-(Trifluoromethyl)phenyl) hydrazinyl)-1,1-difluoro-2-oxoethyl)phenyl) acetamid | | | 50 (38 ± 2) | 26 ± 1 | | | > 100 (pro) | |

(continued)

**Table 1** (continued)

| Inhibitor | Source | Target enzyme | Percentage of inhibition (EC μM) | $K_i$ (μM) | Type of inhibition | *Leishmania* species | Parasite $IC_{50}$ μM (evolutive form) | Reference |
|---|---|---|---|---|---|---|---|---|
| (2-Acetamidophenyl)-*N*-benzyl-α,α-difluoroacetamide | | | 50 (37 ± 6) | 32 ± 3 | | | > 100 (pro) | |
| 1-(4-Bromophenyl)-3-(4-Nitrophenyl)-2-Propen-1-one (**chalcone LC39**) | | | 71.9 ± 11.6 (100) | n.d. | n.d. | *L. infantum* | 398 ± 44.2 (pro)<br>42.3 ± 17.1 (ama) | [151] |
| 1-(4-Methoxyphenyl)-3-(4-Nitrophenyl)-2-Propen-1-one (**chalcone LC41**) | | | 72.3 ± 0.3 (100) | | | | 319.1 ± 14.3 (pro)<br>43.7 ± 13.7 (ama) | |
| Caffeic acid phenethyl amide (**CAPA**) | | | 50 (6.9 ± 0.7) | 3.9 ± 1.0 | Competitive | *L. amazonensis* | 82.56 (pro) | [152] |
| $N^{\omega}$-Hydroxyl-arginine (**LOHA**) | Natural | ARG (cell extract) | 0.3[b] | ~30 | n.d. | *L. major* | n.d. | [83] |
| | | | 0.15[b] | | | *L. infantum* | | |
| DL-α-Difluoromethylornithine (**DFMO**) | Synthetic | ODC (cell extract) | n.d. | 125 | Irreversible | | 38 (pro) | [87] |
| 3-Aminooxy-1-aminopropane (**APA**) | | ODC (cell extract) | ~76 (50) | n.d. | n.d. | *L. donovani* | 42 (pro)<br>5 (ama) | [92] |
| *N*-[4-(2-Oxo-2H-chromen-3-yl)phenyl]-$^{1}$H-1,2,4-triazole-3-carboxamide (**M2**) | | rODC | n.d. | 79.73 | Uncompetitive | | 350 (pro) | [96] |
| 8-[3-(2,5-Dimethylpyrrol-1-l)benzoyl]-3-(4-methoxyphenyl)-1-oxa-8-azaspiro[4.5]dec-2-ene (**M5**) | | | | 370.63 | Non-competitive | | 125 (pro) | |
| Mangiferin | Natural | | | 107.57 | Non-competitive | | 950 (pro) | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 1,4-Diamino-2-butanone (**DAB**) | Synthetic | ODC (cell extract) | 100 (100) | n.d. | n.d. | *L. amazonensis* | ~144 (pro) | [153] |
| Diepoxide naphthoquinonoid (**D17**) | | rODC | ~88 (7) | | Non-competitive | *L. donovani* | 7.2 ± 1.8 (pro)<br>0.18 ± 0.005 (ama) | [97] |
| **CGP40215A** | | AdoMetDC (cell extract) | 0.35 ± 0.04[c] (18) | | n.d. | | 18 (pro) | [100] |
| Hypericin | Natural | rSpdS | n.d. | 3.68 | Mixed | | 18 (pro) | [106] |
| Tomatine | | rTryS | | 12.54 | Competitive | | 18.02 (pro) | [126] |
| Conessine | | | | 3.12 | | | 13.42 (pro) | |
| Uvaol | | | | 3.55 | | | 11.23 (pro) | |
| Betulin | | | | 6.33 | | | 11.71 (pro) | |
| $N^5$-substituted paullone derivative **FS-554** | Synthetic | | 50 (0.349 ± 0.047) | n.d. | n.d. | *L. infantum* | 112.3 ± 1.1 (pro) | [118] |
| $N^5$-substituted paullone derivative **MOL2008** | | | 50 (0.15 ± 0.006) | | Competitive | | 12.6 ± 1.6 (pro) | [128] |
| 4-(4,4,8-Trimethyl-7-oxo-3-oxabicyclo[3.3.1] non-2-yl)-benzoic acid methyl ester (**PS-203**) | | | n.d. | 14.2 ± 0.8 | | *L. donovani* | 4.9 ± 0.4 (pro) | [129] |
| Auranofin | | rTryR | | 0.155 ± 0.035 | n.d. | *L. infantum* | 9.68 ± 1.2 (pro) | [134] |
| | | | | | | *L. major* | 15.66 ± 1.24 (pro) | |
| Chloro(triethylphosphine)gold(I) (CTPAu) | | | | 0.018 ± 0.007 | | *L. infantum* | 16.59 ± 1.03 (pro) | |
| | | | | | | *L. major* | 17.48 ± 1.02 (pro) | |
| $(Cl_2Au(III)(Pbi)Au(I)(PPh_3))(PF_6)$ | | | 97 (0.1) | 0.022 ± 0.011 | | n.d. | n.d. | [135] |

(continued)

**Table 1** (continued)

| Inhibitor | Source | Target enzyme | Percentage of inhibition (EC μM) | $K_i$ (μM) | Type of inhibition | *Leishmania* species | Parasite $IC_{50}$ μM (evolutive form) | Reference |
|---|---|---|---|---|---|---|---|---|
| Ag(0) | | | n.d. | 0.5 ± 0.2 | | *L. infantum* | 2.18 ± 0.33 (pro)[d]<br>1.76 ± 0.24 (ama)[d] | [136] |
| Ag(I) | | | | 0.050 ± 0.01 | | n.d. | n.d. | |
| 4-((1-(4-ethylphenyl)-2-methyl-5-(4-(methylthio)phenyl)-1H-pyrrol-3-yl)methyl) thio-morpholine | | | n.d. | 4.6 ± 2.5 | Competitive | *L. infantum* | 13.77 (ama) | [138] |
| 3,5-dimethyl-4-isoxazolyl selenocyanate (**1h**) | | | 50 (0.46 ± 0.01) | n.d. | n.d. | | 0.73 ± 0.10 (ax ama) | [139] |
| | | | | | | | 23.2 ± 4.3 (ama) | |
| 3,3-(Diselenodiyldimethanediyl)bis(2-bromothiophene) (**2d**) | | | 50 (6.85 ± 0.49) | | | | 1.2 ± 0.03 (ax ama) | |
| | | | | | | | 14 ± 2.1 (ama) | |
| 1,1-(Diselenodiyldimethanediyl)bis(1H-benzotriazole) (**2e**) | | | 50 (> 75) | | | | 0.45 ± 0.03 (ax ama) | |
| | | | | | | | 14.4 ± 2.6 (ama) | |
| 6-(sec-butoxy)-2-((3-chlorophenyl)thio) pyrimidin-4-amine | | | n.d. | 0.25 ± 0.18 | Competitive | | 29.43 (pro) | [140] |
| 2-chloro-6-(phenylthio)pyrimidin-4-amine | | | 30 (10) | 12.0 ± 1.0 | | | 11.0 ± 2.0 (pro) | [141] |
| (E)-1-(2-methoxy-4-((3-methylbut-2-en-1-yl) oxy)phenyl)-3-(4-nitrophenyl)prop-2-en-1-one | | | n.d. | 0.45 ± 0.11 | | *L. donovani* | 3.0 (pro)<br>14 (ama) | [142] |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *N*-(4-bromo-3-methylphenyl)-5-nitrothiophene-2-carboxamide (**C10/7**) | | | 50 (0.19 ± 0.08) | n.d. | | *L. infantum* | n.d. | [143] |
| *N*-{4-methoxy-3-[(4-methoxyphenyl) sulfamoyl]phenyl}-5-nitrothiophene-2-carboxamide) (**A1/7**) | | | 50 (0.52 ± 0.14) | | | | | |
| 2-(diethylamino)ethyl4-((3-(4-nitrophenyl)-3-oxopropyl)amino)benzoate (**compound 3**) | | | 50 (7.5 ± 2.5) | | | | 12.44 ± 1.09 (pro) | [144] |
| $N^1$-((1-(4-(4-(2-(Naphthalen-2-yl)ethyl)thiazol-2-yl)-3-(2-(2-oxoimidazolidin-1-yl)ethoxy) phenyl)-1H-imidazol-2-yl)-methyl)ethane-1,2-diaminium 2,2,2-Trifluoroacetate (**3e**) | | | 50 (5.1 ± 0.4) | | n.d. | | 12.8 ± 0.7 (pro) 12.8 ± 1.3 (ama) | [148] |
| $N^1$-((1-(4-(4-(2-([1,1′-Biphenyl]-4-yl)ethyl) thiazol-2-yl)-3-(2-(2-oxoimidazolidin-1-yl)eth-oxy)phenyl)-1H-imidazol-2-yl)methyl)ethane-1,2-diaminium 2,2,2-Trifluoroacetate (**3f**) | | | 50 (8.6 ± 1.4) | | | | 5.3 ± 0.3 (pro) 5.3 ± 0.2 (ama) | |

ARG, arginase; rARG, recombinant arginase; ODC, ornithine decarboxylase; rODC, recombinant ornithine decarboxylase; AdoMetDC, S-adenosylmethionine decarboxylase; rSpdS, recombinant spermidine synthase; n.d., not determined; pro, promastigote; ama, amastigote; ax ama, axenic amastigote.

Acronyms or substances' codes used in the original articles are highlighted in bold

[a]Result expressed as μmol/μg protein

[b]Result expressed as mU/$10^7$ parasites

[c]Result expressed as nmol $h^{-1}$(mg protein)$^{-1}$

[d]$IC_{50}$ values for Ag(0) nanoparticles encapsulated by ferritin molecules

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# Nano and Microstructured Delivery Systems for Current Antileishmanial Drugs


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## Contents



**Abstract** Current treatment of both cutaneous and visceral leishmaniasis requires multiple injections with toxic drugs that cause severe adverse effects. Unfavorable pharmacokinetics and biodistribution, together with difficulty in gaining intracellular access, contribute to the lack of adequate therapies. In this context, drug delivery systems based on micro and nanotechnologies have arisen as promising tools to improve drug absorption, bioavailability, chemical and physical stability, and cell targeting. These factors could be particularly useful for leishmaniasis treatment, as they can be endocytosed by macrophages, the host cell of the parasite, sparing sophisticated targeting functionalization. Here, the main advantages, drawbacks,

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and perspectives in using different particulate delivery systems for a more effective and safer leishmaniasis treatment with the current approved drugs will be discussed.

**Keywords** Chemotherapy, *Leishmania*, Liposome, Microparticles, Nanoparticles

# 1 Introduction

## *1.1 Leishmaniasis*

Leishmaniasis is a group of vector-borne diseases caused by protozoan parasites of the genus *Leishmania,* which are transmitted to mammalian hosts by the bite of infected female sandflies [1]. The disease is classified by the World Health Organization (WHO) as a Neglected Tropical Disease (NTD), because it primarily affects low-income countries and consequently is considered low priority for international public healthcare efforts, leading to insufficient prevention and inadequate treatment options [2].

There are two main clinical manifestations of leishmaniasis: cutaneous (CL) and visceral (VL). Epidemiologically, the disease is endemic in about 98 countries around the globe, and official reports state approximately 0.7–1 million new cases of CL and 50,000–90,000 cases of VL in 2017, with approximately 1 billion people at risk of infection [1–3].

Protozoans parasites of the *Leishmania* genus are unicellular eukaryotic cells with a digenetic life cycle and two distinct life stages: the promastigote form in the insect vector and the amastigote form in the mammalian host [4]. During its life cycle, the parasite, in its promastigote form, infects cells of the mammalian host, including macrophages, neutrophils, and dendritic cells [4]. Once inside, the parasite begins to differentiate into the amastigote form within the phagosome compartment, which becomes the parasitophorous vacuole [5].

The clinical manifestation of leishmaniasis depends on the parasite species and the host's immunological status [6] and is marked by the presence of intracellular amastigotes in target tissues and organs; in the skin and mucosa for CL and in the spleen, liver, lymph nodes, and bone marrow for VL [1]. The most serious clinical form is VL, also known as kala-azar/calazar. In VL, the parasite species *Leishmania donovani* and *L. infantum* (syn *L. chagasi*) infect macrophage-rich internal organs, such as the spleen, liver, and bone marrow and can be fatal in more than 95% of untreated cases [7]. CL is the most prevalent clinical form of the disease, which is characterized by localized and slow-growing skin ulcers on exposed parts of the body [1]. In some cases, CL progresses to a more severe presentation with multiple lesions, diffuse cutaneous leishmaniasis (DL), and disseminated cutaneous leishmaniasis (DCL), or a mucosa-mutilating form, mucosal leishmaniasis (ML). CL is associated with serious negative psychological and social repercussions for the patient, due to the scarring and mutilation left by the disease, which can affect the

patient for the rest of their life [8]. Risk factors for this progression include the patient's immunological and nutritional status, the *Leishmania* species, and the lack or inadequacy of treatment [9]. Therefore, complete treatment and at an early stage is essential.

## *1.2 Current Chemotherapy*

Notwithstanding the economic and social impact of the disease, there is as yet no approved vaccine against *Leishmania* infection in humans [10]. The existence of more than 20 parasite species causing the different forms of the disease in humans, each with distinct gene expression, in addition to the intracellular location of the parasite, parasite eukaryotic cell complexity, and the lack of an appropriate adjuvant, has hindered the development of an effective vaccine. Likewise, the development of innovative medicines is extremely problematic for leishmaniasis, as active drugs need to be of broad spectrum, have appropriate bioavailability to reach infected organs, and be able to permeate multiple membrane barriers to access the intracellular amastigote forms within the parasitophorous vacuole [11].

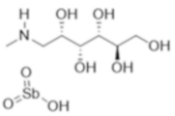
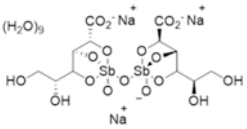
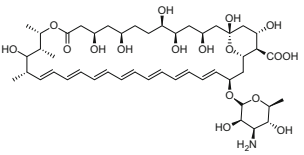
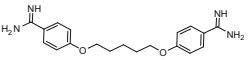
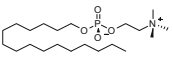
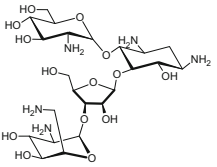
Current disease control relies on a limited number of old and repositioned drugs. Pentavalent antimonials like meglumine antimoniate (e.g., Glucantime) and sodium stibogluconate (e.g. Pentostam) are the first-choice drugs in many countries, while amphotericin B (AmB) and pentamidine are used as second choices, and miltefosine and paromomycin are the final options [12]. All available treatments have one or more drawbacks, such as administration by intramuscular or intravenous injections, severe toxic side effects, variable efficacy, prohibitive price, and resistance development [13].

This inadequacy of treatment is very worrying, especially in the treatment of CL, as although the disease is localized to the skin and does not cause death, the serious systemic effects caused by the drugs can cause the patient to abandon the treatment, which increases the chance that the disease could worsen into DL or DCL. For this reason, the WHO and DNDi (arm of the Médecins Sans Frontières, focused on the development of new drugs for NTDs) have prioritized the search for new oral and local treatments for CL in the case of disease with up to four ulcers of a maximum of 3 cm in diameter, which accounts for >90% of CL cases [14].

However, the development of oral and local treatments for leishmaniasis is complicated, as the available drugs have inadequate physicochemical properties, as depicted in Table 1. In general, most of the molecules display negative Log*P* values ($0 < \text{Log}P < 3.0$, ideal range), molecular weights greater than 500 Da and high topological polar surface areas (TPSA), which are not suitable for cell membrane permeation or skin and intestinal absorption [15]. Thus, the properties of these drugs in their current state hinder their use by oral and topical routes [14].

Treatment via the oral route is an attractive option and often the priority when new therapies are considered. However, in addition to all the aforementioned issues, the intestinal barrier is also a significant factor for this route of treatment, once the

**Table 1** Chemical properties of current antileishmanial drugs

| Drugs | Chemical structure | Molecular weight | Log*P* | pH | TPSA |
|---|---|---|---|---|---|
| Meglumine antimoniate (Glucantime®) |  | 365.98 | −3.09 | 11.32 | 141.9 |
| Sodium stibogluconate (Pentostan®) |  | 907.89 | −2.03 | 1.62 | 260.59 |
| Amphotericin B |  | 924.10 | −2.14 | 6.68 | 319.61 |
| Pentamidine |  | 340.42 | 3.24 | 11.83 | 118.20 |
| Miltefosine |  | 408.58 | 0.08 | 4.85 | 55.76 |
| Paromomycin |  | 615.63 | −5.55 | 11.32 | 347.32 |

*TPSA* topological polar surface area. Data obtained from the ADMET Predictor (Version 8.1.0.11. Simulations Plus, Lancaster, CA, USA)

molecules need to cross this barrier to reach the organism. An oral miltefosine treatment has been reported to have variable efficacy for different *Leishmania* species in the Americas, which prevents its use in that continent, despite being the only approved drug for oral VL treatment in South Asia [16]. Patient adherence to treatment must also be taken into account, as the patient would be responsible for completing the treatment regime themselves. Drug resistance could arise if the regime is not strictly followed, which could also reduce efficiency and hinder further attempts with new therapies [17].

Thus, the most appropriate route for the treatment of CL is the local one. However, the drug limitations and inadequacy have been associated with the lack of effectiveness of the paromomycin-based cream in the Americas, despite being used in the Middle East for infections caused by *L. tropica* and *L. major* [18]. In addition, low effectiveness (30%) was reported for a 3% AmB-based cream

(Anfoleish) during a clinical study (phases Ib, II) in Colombia for patients with *L. braziliensis* and *L. panamensis* [19].

To circumvent the problem of low skin permeation, some doctors in Iran and Brazil have injected Glucantime directly under the skin via the subcutaneous route. However, due to the chemical characteristics of this drug, it diffuses rapidly into the bloodstream requiring repeated injections (1–2 injections per week for 3–7 weeks). Thus, this requirement of repeated and painful local injections translates to low adherence by patients, particularly children, especially as the side effects, which include anaphylactic shock, are not effectively reduced [20].

Thermotherapy has emerged as a non-medication alternative for the local treatment of CL. It consists of the application of radiofrequency waves (+ 50°C) on the edge of the ulcer, curing 70% of the cases in Colombia [21], reaching 86% in Brazil [22]. The problem is the frequent reports of burns due to the high temperature, which is torturous for children. In addition, to ensure effectiveness, it must often be associated with conventional drug treatments.

Another issue with the conventional treatment is failure due to emerging resistance; in this case, drug combination has been a promising strategy adopted in recent clinical trials. For instance, on clinicaltrials.gov there are CL treatments with pentavalent antimonials associated with miltefosine, liposomal AmB, pentoxifylline, allopurinol, or paromomycin. Drug association for topical CL treatment has also been employed, like paromomycin with pentoxifylline, imiquimod or thermotherapy (clinicaltrials.gov). Although drug combination may be useful for reducing resistance and increasing treatment efficacy, it does not solve the physicochemical limitations of the drugs.

Another straightforward strategy is drug repositioning or repurposing, where new uses for available approved drugs or drug candidates are discovered and refined. The main advantage of this approach is that many regulatory phases required for a new drug candidate to gain approval can be bypassed, which shortens time and reduces costs on drug discovery pipelines [23]. Another more technological approach to counteract the limitations of new and old antileishmanial drugs is the use of drug delivery systems (DDS).

## *1.3 Drug Delivery Systems (DDS)*

The first works published employing nano and microsystems to protect and improve the stability of different types of hydrophilic and lipophilic compounds have attracted the interest of pharmaceutical scientists to apply them as DDS. DDS are pharmaceutical technologies/formulations used to bypass issues related to drug solubility in biological fluids, increase permeation, promote targeting, and protect the drug against physical, chemical, and/or enzymatic degradation [24]. Widespread interest and research on this topic have improved DDS greatly and now a single system can carry more than one active and release them independently under specific conditions, such as temperature or pH increase. This has enabled the use of

combination therapies with greater chances of success. Moreover, DDS may target the drug to an appropriate organ or cell type and allow administration through desirable non-invasive routes, such as oral and topical, improving patient compliance, and the treatment success rate [17]. In addition, DDS have been studied in order to optimize the safety/efficacy ratio in delivering the required amount of drug to the target, thereby reducing the number of doses and amount of drug per dose, which consequently decreases the treatment costs [17].

The use of DDS to create new strategies and develop new treatments against *Leishmania* infection has garnered interest. The fact that DDS have the potential to reach the intracellular location where the parasites reside is a particularly valuable aspect for leishmaniasis treatment. Thus, phagocytic macrophages from skin in CL or visceral organs in VL can uptake drug-loaded nanoparticles or microparticles and deliver the active molecule directly to the parasite, reducing toxicity related to drug accumulation in off-target organs [24]. However, much remains to be done, as there are not yet enough studies with this technology in the literature. A simple search on the *Web of Science* database using "drug AND delivery AND system" as descriptors in February 2020 found 93,908 publications, although only 313 were related to *Leishmania* ("*Leishmania** AND drug AND delivery AND system"). This is corroborated in clinical trial studies (clinicaltrials.gov), since of the 149 identified for the leishmaniasis treatment only 8 used DDS, 4 of those being AmBisome that is already an approved DDS.

Of all the available nano and microsystems, the most studied for leishmaniasis treatment can be divided into (1) lipid-based nanosystems, (2) polymeric systems, (3) dendrimers, (4) cyclodextrins, (5) carbon nanotubes, and (6) metallic nanoparticles (Fig. 1). However, even today the most used for biological applications are lipid-based nanosystems and polymeric nanoparticles, as evidenced by their predominance in the 51 nanosystems approved by the US Food and Drug Administration (FDA), and in the 77 DDS in clinical trials in 2016 [25].

Lipid-based systems include liposomes, nano and microemulsions, solid lipid nanoparticles and nanostructured lipid carriers. In general, liposomes are still the most studied and used. Liposomes were first described in the 1960s and were the pioneering nanosystems in studies on drug and protein encapsulation. Liposomes are composed of a single or multiple phospholipid bilayers that self-organize in aqueous medium, analogous to the cell membrane, and were the first clinically approved nanosystem by FDA in 1995 (Doxil) [26]. The advantages of this system are attributed to its biocompatibility, easy preparation and functionalization, maintaining the drug availability in the bloodstream for a long period of time, and ability to encapsulate and protect hydrophilic and lipophilic drugs, since it has both an aqueous nucleus and a lipid membrane in its structure [26].

The tropism and accumulation of liposomes in the liver and spleen make them an appealing system for VL treatment. Conventional liposomes are preferably captured by the liver's macrophages, allowing the drug to be released into the target organ, reducing the adverse effects of treatment [27]. Moreover, surface modifications of these systems with different parasite or human target ligands recognized by the

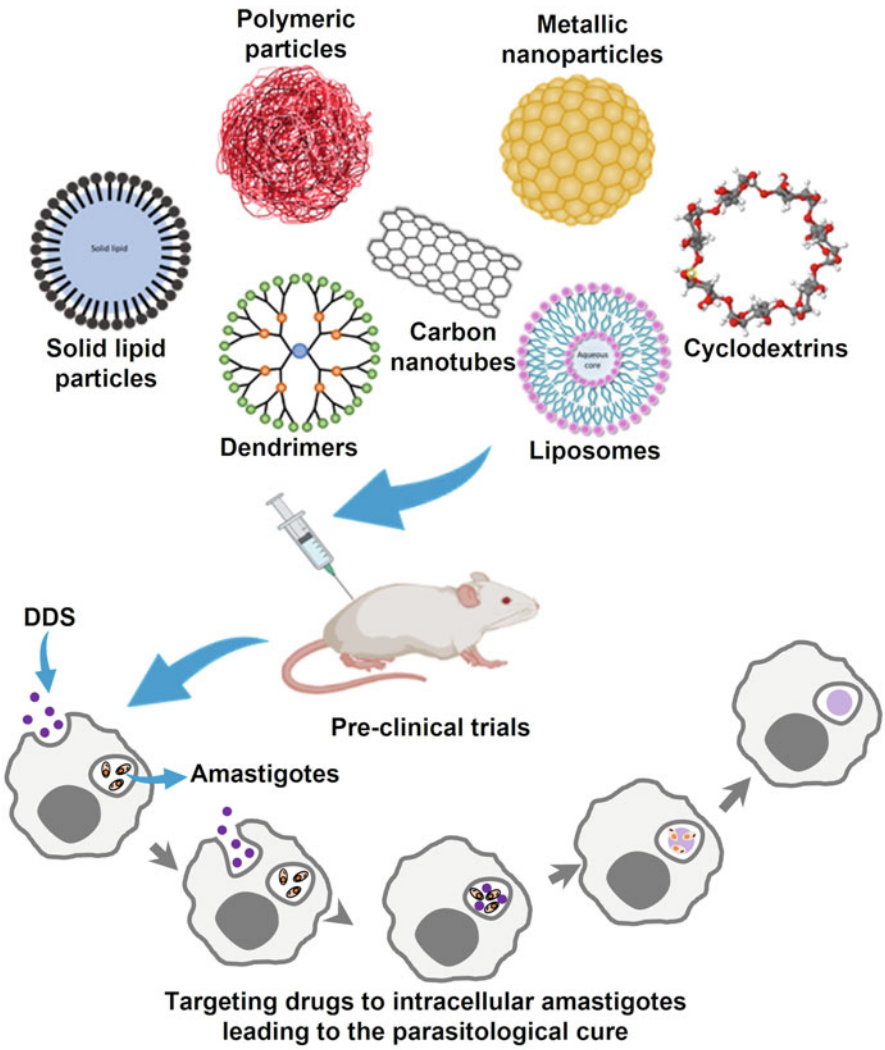



**Fig. 1** Most studied DDS for leishmaniasis treatment and its uptake by infected macrophages. Schematic representation of the main systems in preclinical trials for leishmaniasis' treatment. DDS are internalized by macrophages, resulting in drug release directly into the main cells infected by *Leishmania*, leading to parasitological cure

receptors present on the surface of the macrophages are still being studied in order to further enhance their uptake by macrophages [28].

Liposomes loaded with AmB (AmBisome) were the first DDS approved by the FDA in 1977 for the treatment of leishmaniasis and until now AmB is still the main drug investigated for use in these systems. This liposomal formulation showed less toxicity and greater efficiency (>90%), when compared to the conventional formulation of AmB in patients [28]. On the other hand, the prohibitive cost restricts

AmBisome use to severe or unresponsive cases of VL and MCL only. Other disadvantages of the liposomes include low stability in variable temperature and pH, which can occur during storage and transportation of drugs [27].

In this sense, since 1990 solid lipid nanoparticles (SLNs) have become an alternative to liposomes as a lipid nanosystem. SLNs are aqueous heterogeneous dispersions with low viscosity composed of a solid lipid matrix stabilized by a biocompatible surfactant used as an emulsifier at the external aqueous phase [29]. This carrier system is considered more stable and cheaper than liposomes, and safer than polymeric materials due to the lack of organic solvents used during their production. However, SLNs have some disadvantages including tendency for gelation, encapsulation of a limited number of lipophilic molecules, and low incorporation rates of others compounds due to the crystalline structure of the solid lipid [30]. To overcome this limitation nanostructured lipid carriers (NLCs) have emerged, which differ from SLNs by the core lipid composition. Drug encapsulation in NLCs enhanced the physical stability of the drug, which improved targeted drug delivery and release kinetics as well as the capacity to protect the drug from expulsion during its storage [29, 30]. Nevertheless, problems with NLCs are the partial coalescence that may occur between a partly crystalline particle and the liquid oil portion of another particle. Factors including the types of lipids, emulsifiers, particle size, and lipid ratio are important for this coalescence event [31].

Polymeric systems, like nanoparticles, microparticles, and polymeric micelles, are another form of DDS. In general, they may be produced in different sizes (from nano to microsizes) and shapes through the use of different biocompatible and biodegradable polymers individually or in blends. They are also stable and can be administered through most routes [17, 32]. In addition, these polymers can also be modified chemically or physically to allow greater interaction with the drug, controlling drug release for long periods (up to months) or under specific conditions such as by enzymatic degradation, temperature or pH variation, and finally to vectorize the drug for a specific tissue or cell type [17, 33–36].

Currently, the most used polymers for this purpose are the natural polymers albumin, chitosan, alginate, and gelatin, and the synthetic polymers, polycaprolactone (PCL), poly(lactic acid) (PLA), poly(lactic acid-co-glycolic acid) (PLGA), and polyacrylates (PCA) [28, 37]. As mentioned previously, polymers can be organized in different forms, which are differentiated by their chemical and organizational characteristics, for example particles and micelles.

The polymeric particles can be prepared in nano or micro size (nanoparticles and microparticles, respectively) and can be organized in the form of capsules or spheres. In nano or microcapsules, the polymer forms a polymeric membrane that encapsulates a core, usually oily. In this case, the drug can be incorporated in the core or dispersed into the polymeric capsule. Nano or microspheres are composed of a dispersed polymer matrix and the compound is spread homogeneously or heterogeneously in this matrix [28]. Polymeric particles have the advantage of controlling the release of drugs, being able to incorporate hydrophilic and hydrophobic drugs, higher stability than lipid formulations, and the possibility of choosing a biocompatible and/or biodegradable material. However, the DDS scaling-up production

remains a challenge due to the lack of a simple and reproducible methods for all different polymeric particles.

Finally, micelles are composed of amphiphilic copolymers (structures made from two or more monomers) at or above the critical micellar concentration in which the hydrophobic inner core is surrounded by a hydrophilic shell [38]. The great solubility of highly lipophilic actives and controlled drug release make micelles another interesting system. However, as only lipophilic drugs can be used in the application, the low capacity of drug and the dependency on the critical micelle concentration are crucial disadvantages of polymeric micelles [39].

Dendrimers are another type of polymeric system. They are highly branched supramolecular structures composed basically of three architectural components: a multifunctional central core (nucleus), branched units, and surface groups [40]. The dendrimers synthesis can follow the divergent or convergent method. In the divergent method, the dendrimer grows from a nucleus, followed by ordered reactions that promote homogeneous and concentrated growth of the structure, each set of reactions its known as a new generation.

Fast synthesis, highly symmetric products and of high generation, and the possibility of surface functionalization are favorable characteristics obtained by this divergent method. Meanwhile, the occurrence of defects in higher generation dendrimers in addition to the excess of reagents and the number of chemical processes needed to form a large structure are limiters of the divergent method [40, 41]. The convergent method of synthesis is through the union of previously prepared branched structures. This method confers better control of the structure than the divergent method since the occurrence of side reactions is reduced, in addition to reducing the number of reagents used and to obtaining purer compounds [40]. In contrast, difficulties in the production of high generation dendrimers and in the modification of the terminal groups are clear disadvantages of this process [41].

The branched structure of the dendrimers provides many free active sites that can be used for precise and controlled reactions, the cavities inside can be used to encapsulate molecules of different sizes, and their controlled synthesis allows specific groups to be added at the ends facilitating their vectorization and modification for different applications [42, 43]. When compared to liposomes, dendrimers are more favorable due to their greater stability and the possibility of covalently binding to actives. When compared with linear polymers, dendrimers have the advantage of better biodistribution and pharmacokinetics for biomedical application, higher homogeneity, and the ability to alter their solubility and targeting properties due to association with ligands [40]. However, there are drawbacks of dendrimers such as the high cost of production, difficulty in functionalizing the surface, toxicity generated by many reactive sites in the structure, and the complexity in forming high generation dendrimers due to the occurrence of steric impediment, which limit the applications of this DDS [40].

Cyclodextrins (CDs) are another example of supramolecular structures with interesting properties for use as DDS [44] CDs are cyclic structures with a hydrophobic cavity that can be loaded with many actives forming inclusion complexes and a hydrophilic exterior allowing solubility in aqueous medium, with the aim of

increasing the solubility and bioavailability of the drugs, as well as improving their oral efficacy [44, 45].CDs are water-soluble structures, biodegradable, nontoxic and most of them are formed by six, seven, or eight glucose units (α, β, and γ-cyclodextrin, respectively). CDs can be administrated as oral, nasal, ocular, rectal, or dermal formulations. However, care must be taken when CDs are administrated by the parenteral route as nephrotoxicity and hemolysis can be caused by precipitation in the kidneys [46].

Inorganic nanosystems have attracted increasing attention for drug delivery applications. Although in most cases they are not biodegradable, the biocompatibility and attractive properties like easy preparation, good physicochemical characteristics, high cellular uptake, and decent storage stability have justified their potential use and meant that these DDS have been more explored in the last few years in the development of new treatments and innovative diagnostic systems [47, 48]. Some examples of inorganic nanosystems for biological applications are carbon nanosystems, metallic nanoparticles, natural clay nanoparticles, mesoporous silica nanoparticles (MSNs), layered double hydroxides (LDHs), and quantum dots (QDs) [47].

In this sense, the research into the use of carbon nanotubes (CNTs) as DDS has increased because of their positive characteristics and properties, such as large surface area, high aspect ratio, and higher loading capacity. CNTs are literally tubes made of carbon with a nanometric diameter, studied in the health field as DDS mainly for cancer treatment, for transferring genetic material in gene therapies and as biosensors. For leishmaniasis treatment, AmB can be attached to functionalized CNTs presenting higher efficacy than free drug [28]. These systems are particularly useful for drugs with low solubility in water, like AmB. Thus, the conjugation of the drug to CNTs aims to create formulations capable of increasing solubility, reducing aggregation, increasing cellular uptake, and consequently enhancing therapeutic effects and reducing the toxic effects of the drug [49, 50]. The most worrying problem associated with these systems is the fact that they are non-biodegradable, therefore, the biological fate does not behave in the same way as the administration systems previously mentioned. In addition, there are still not enough results regarding the toxicity of CNTs within the body, thus extensive research is still required to guarantee their safety profile for drug delivery [50].

Metallic nanoparticles are also appealing for leishmaniasis treatment due to their magnetic, optical, and plasmonic properties. The use of magnetic iron oxide nanoparticles exposed to a pulsed magnetic field to generate heat in a controlled and localized way has been researched for a long time, principally for cancer treatment. The superficial modification of these nanoparticles with immunoglobulins allows them to reach only the cancer cell in the target tissue, which significantly increases the effectiveness of anti-tumor drugs and reduces their toxicity. Recently, magnetic hyperthermia has been applied as an alternative to conventional thermotherapy for the local treatment of CL in order to avoid burns associated with the use of radio frequency waves [51].

In addition to magnetic iron oxide nanoparticles, metallic nanoparticles based on gold, silver, and selenium also have great potential in the treatment of leishmaniasis, since they have been shown to have antileishmanial activity and are biocompatible [52–55]. However, these systems have a limited capacity for conjugation with drugs and high toxicity in macrophages, which has limited their clinical use [52].

## *1.4 How DDS Entries into Cells*

DDS vary enormously in composition, superficial charge, shape and size, which affect the way by which DDS are internalized by macrophages as well as their intracellular fate [53, 54]. The understanding of these characteristics is essential during the process of choosing the appropriate DDS to deliver the drug to the parasitophorous vacuole containing the *Leishmania* parasites in the target organ.

Due to their nanometric sizes, DDS can interact with cells similar to how proteins and virus particles do, and this recognition is meditated by cell surface receptors or directly with the plasma membrane [54]. Macrophages belong to a group of immunological cells responsible for recognizing and degrading pathogens and extracellular material. To play this role, macrophages express pattern-associated recognition receptors (PRRs) on their membrane, these receptors are extremely efficient at recognizing antigenic pathogen surface patterns, particulate material, and damaged self-cells [55]. PRRs include Toll-like receptors (TLRs), scavenger receptors (SRs), mannose receptors (MRs), and Fc receptors (FcRs). The uptake process of DDS most often involves MRs and FcRs [54]. In this context, DDS functionalization has been employed to increase their cellular uptake, as demonstrated by Esfandiari et al. [56] and Afzal et al. [57] using mannose to improve intracellular delivery of paromomycin encapsulated in chitosan nanoparticles or thiolated PLGA nanoparticles, respectively.

After being recognized by the cells, DDS will mostly be internalized via endocytic pathways (Fig. 2). The endocytic process can be divided based on the size and composition of the vesicle; phagocytosis is related to large vesicles (up to 10 μm) while pinocytosis occurs for small size vesicles (up to 5 μm) [58]. Pinocytosis can be further classified into four categories: macropinocytosis (0.5–5 μm), clathrin-mediated endocytosis (100–350 nm), caveolin-mediated endocytosis (20–100 nm), and clathrin- and caveolin-independent pathways (<90 nm) [58, 59].

Phagocytosis occurs in phagocytic cells such as macrophages, this phenomenon is described as a pseudopodal internalization of large bodies with actin remodeling, commonly associated with FcRs and complement receptors (CRs) [54, 59]. While macropinocytosis (MP) is initiated by growth factors which activate tyrosine kinase receptors (RTK). This activation triggers formation of actin-driven membrane ruffles that culminate in the internalization of extracellular fluid and cell debris in a non-specific way [59, 60].

Clathrin-mediated endocytosis (CME) is the process responsible for the internalization of biomolecules including low density lipoprotein (LDL) by the LDL

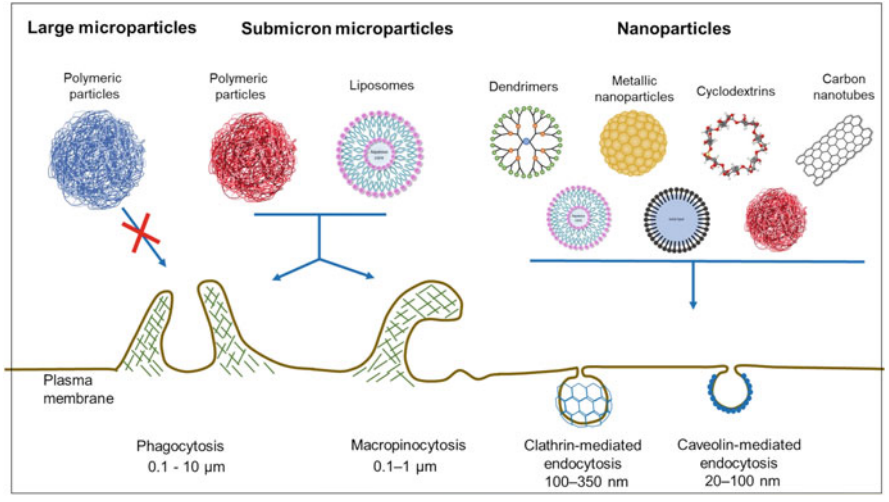



**Fig. 2** Influence of size on the DDS internalization mechanism. Large polymeric microparticles (>10 µm) cannot be internalized by the cells. While submicron microparticles (polymeric particles and liposomes) may be endocytosed by the cells through phagocytosis and macropinocytosis pathways. Lastly, nanosized DDS (300 nm) (dendrimers, metallic nanoparticles, cyclodextrins, carbon nanotubes, polymeric particles, and liposomes) will reach the intracellular environment mainly by clathrin-mediated and caveolin-mediated endocytosis

receptor, or iron by transferrin receptor. The interaction between the ligand and receptor triggers their engulfment in a pit coated with cytosolic proteins mainly clathrin-1 [53]. PRRs including SRs, MRs, and TLRs are related to the CME process [61]. Whereas caveolin-mediated endocytosis is characterized by flask-shaped plasma membrane invaginations, clustering of lipid rafts containing extracellular contents and coated by proteins called caveolins [65]. Unlike phagocytosis, MP, and CME vesicles that fuse with lysosomes for further processing, caveolin vesicles are transported to the Golgi apparatus or are excreted from the cell [54].

Lastly, internalization of distinct cargo in a clathrin- and caveolin-independent manner (CIE) has been described. CIE pathways are dependent on and classified by the molecular effector required, including Arf6-dependent, flotillin-dependent, Cdc42-dependent, and RhoA-dependent. These pathways are responsible for the internalization of interleukin-2, extracellular fluid, glycosylphosphatidylinositol (GPI)-linked proteins, and growth hormones, among others [53, 59].

Due to the complexity of DDS cellular uptake, many physicochemical features can interfere in this process. The first feature is the size of the DDS, it is commonly accepted that particles with reduced sizes are more efficiently internalized, as extensive membrane and actin rearrangement is necessary to internalize larger particles [53, 62]. Nanoparticles with sizes ranging from 250 nm to 3 μm are more likely internalized by phagocytosis [63]. Whereas particles up to 100 nm in diameter enter through CME, or by caveolin-mediated endocytosis for particles up to 500 nm, as reported by Rejman and coworkers in melanoma B16 cells [62].

In addition to size, another factor to be considered is the particle charge. It is largely accepted that cationic nanoparticles are internalized more efficiently than neutral and anionic particles, due to the negative charge on the cell surface [64]. Additionally, some studies suggest an influence of the surface charge on the internalization pathway, it seems that cationic nanomaterials are internalized by CME and MP, while anionic DDS are more likely to gain entry through a caveolin-mediated endocytosis [65].

Furthermore, the particle shape can play a role. Chithrani et al. demonstrated that spherical gold NPs were more efficiently internalized than rod-shaped ones [66]. Simulations indicated that the uptake ratio would be higher for spheres followed by cubes, rods, and discs based on the energy required for membrane bending [67]. Regarding the membrane bending energy, more rigid nanoparticles enter more efficiently than softer nanoparticles [68].

It is important to mention that the studies referenced in this review represent just a few works found in the literature. This subject has been extensively studied by many groups from different research areas and has generated conflicting results, highlighting the complexity of this process and how the nanomaterial properties can actively affect it.

## 2 DDS for Leishmaniasis

In this chapter, we will explore, through some examples in the literature, the main advantages, drawbacks, and perspectives of using distinct DDS incorporating current antileishmanial drugs tested against in vivo models of leishmaniasis. Despite the availability of several reports employing DDS loaded with new antileishmanial drug candidates, such as chalcones [69–71], quercetin [72], buparvaquone [73], and nitroimidazole [74], we focused on approved drugs as the development steps to reach the market will be much cheaper and faster.

### *2.1 Antimonials*

The pentavalent antimonials (Sb), meglumine antimoniate (MA) and sodium stibogluconate (SSG), have been employed as the first-line treatments for CL and VL worldwide since the 1920s [75]. Their mechanism of action is still not well understood, some reports attribute the anti-Leishmanial activity to the inhibition of enzymes related to DNA synthesis [76], zinc-finger proteins [75], and redox balance, such as trypanothione reductase [77]. Despite their effectiveness, an increase in therapeutic failure began to be noticed in the beginning of the 1980s [78] and an expressive emergence of parasite resistance resulted in the abandonment of antimonials as the first-choice treatment for VL in India, a highly endemic region [79]. Other drawbacks related to Sb treatment include the long treatment regimen, parenteral administration routes, and severe side effects, including myalgia, pancreatitis, pancytopenia, hepatotoxicity, and cardiotoxicity [75].

In Table 2 all the most relevant publications regarding encapsulation of pentavalent antimonials in DDS with in vivo results are listed. In general, liposomes lead as the preferred DDS for antimonials, with the aim of reducing the number of doses or changing the administration route to increase patient compliance and therapeutic success. A single dose of SGG in liposomes containing stearylamine was able to reduce *L. donovani* parasite load in the liver, spleen, and bone marrow of BALB/c mice at a dose 25 times lower than of the free drug [80] Similarly, a mixture of conventional and pegylated liposomes containing MA significantly reduced the detection of *L. infantum* DNA in the liver and spleen of BALB/c mice after a single dose at 20 mg/kg, when compared with free drug at the same dose [81]. It has been reported that conventional liposomes enclosing SSG reduced lesion growth caused by *L. mexicana* or *L. major* in BALB/c mice after intralesional or intravenous administration for 5 days at 50 mg/kg/day [82]. In contrast, intralesional treatment with MA-loaded liposomes [83] or nanohybrid hydrosols [84] was not very efficient in controlling CL lesion growth caused by *L. amazonensis* or *L. major* in hamsters or mice respectively, although intralesional Sb is already clinically used to treat CL.

In an attempt to establish the use of antimonials by a non-invasive route, MA was encapsulated in conventional or stearylamine-liposomes [85, 86]. Both formulations

**Table 2** Pentavalent antimonial-loaded DDS

| Pentavalent antimonials | | | | | | | |
|---|---|---|---|---|---|---|---|
| Route | Nanosystem | Size (nm) | Parasite | Model | Regimen | Efficacy/outcome | Refs. |
| IV | Stearylamine-bearing liposome (SSG) | ND | *L. donovani* | BALB/c mice | 12 mg/kg – single dose | When compared to free drug at 300 mg/kg, liposomal antimony suppressed parasitic load by 93%, 98%, and 84% in liver, spleen, and bone marrow, respectively, | [80] |
| IV | Mixture of conventional and pegylated liposome (MA) | 207–229 | *L. infantum* | BALB/c mice | 20 mg/kg – Single dose | 28 days after treatment with liposomal antimony 0% (spleen) and 41% (liver) of parasite DNA was detected by qPCR, in comparison with 25% (spleen) and 83% (liver) for free drug | [81] |
| IV or IL | Conventional liposome (SSG) | ND | *L. mexicana* / *L. major* | BALB/c mice | 50 mg/kg/day for 5 consecutive days | For both parasites, the IL route was more effective at controlling lesion growth | [82] |
| IL | Nanohybrid hydrosols – VSb ($SbCl_5$) | 35–45 | *L. amazonensis* | Syrian hamsters | 4.09 or 0.65 mg/mL/day for 3 weeks | Irrespective of the dose, IL VSb were 1.8-fold more efficient than free drug given by the same route | [84] |
| IL | Conventional liposome (MA) | 123–138 | *L. major* | BALB/c mice | 139 μg/mL 2× week for 35 days | After treatment no significant parasite inhibition | [83] |
| Topical | Stearylamine-bearing liposome (MA) | 182 ± 17 (0.5%) 286 ± 48 (1%) 760 ± 159 (2%) | *L. major* | BALB/c mice | 50 mg of formulation at 0.5, 1, or 2% Sb, 2×/day for 4 weeks | 4 weeks after treatment liposomal groups exhibited significantly smaller lesion size and lower parasite burden compared to groups treated with free drug | [85] |
| Topical | Conventional liposome (MA) | 113 ± 1 | *L. major* | BALB/c mice | 50 mg of formulation at 6.4% Sb, 2×/day for 4 weeks | 4 weeks after treatment the topical treatment induced lesion size reduction and low spleen parasite burden ($P < 0.001$) in comparison with empty liposomes | [86] |

(continued)

**Table 2** (continued)

| Pentavalent antimonials | | | | | | | |
|---|---|---|---|---|---|---|---|
| Route | Nanosystem | Size (nm) | Parasite | Model | Regimen | Efficacy/outcome | Refs. |
| Oral | Beta-cyclodextrin (MA) | ND | *L. amazonensis* | BALB/c mice | 32 mg Sb/kg/day on days 10–16 and 31–36 of infection | After 80 days of infection, the effectiveness of oral treatment was equivalent to free drug (IP) but at a twofold-lower Sb dose | [87] |
| Oral | Polarity-sensitive nanocarrier (SbL8) | 100–300 | *L. amazonensis* | BALB/c mice | 200 mg Sb/kg/day for 30 days | The treatment with Sb formulation was capable of reducing the lesion parasite burden, compared to free drug in water (oral) and Glucantime (IP) | [88] |

*SSG* Sodium stibogluconate, *MA* Meglumine antimoniate, *Sb* Antimony, *IV* Intravenous route, *IL* Intralesional route, *ND* Not described

were given topically twice a day for 4 weeks for lesions caused by *L. major* in BALB/c mice. In the two studies, liposomes carrying Sb diminished the lesion sizes as well as parasite burden in comparison with mice treated with the free drug or the empty liposomes [85, 86]. By the oral route, beta-cyclodextrin and polarity-sensitive nanocarriers made the administration of MG possible in a CL murine model of *L. amazonensis* [87, 88]. At a dose of 32 mg Sb/kg/day, beta-cyclodextrin nanocarriers were administered orally for 7 days then on days 10 and 31 after infection. After 80 days post-infection the oral treatment efficacy was similar to that of the free drug administered by the intraperitoneal route [87]. Likewise, a polarity-sensitive nanocarrier containing Sb reduced both lesion size and parasite load, at 200 mg Sb/kg/day for 30 days when compared with the free drug administered by the oral route or intraperitoneally [88].

## *2.2 Amphotericin B (AmB)*

AmB is a polyene antibiotic currently considered the most effective drug for leishmaniasis treatment. The mode of action is related to its capacity to bind ergosterol in the parasite membrane leading to membrane perturbation and cell death [89]. It is the first choice for patients refractory to antimonials, pregnant women, and in countries where the antimony resistance is widespread, such as in India [12]. The high efficacy and absence of resistance of this drug are related to the strong interaction between AmB molecule and parasite ergosterol. However, non-specific interaction with mammalian cell cholesterol and the presence of sodium deoxycholate in the formulation, required for drug solubilization, leads to several adverse effects, such as nausea, vomiting, fever, hypopotassemia, anemia, and cardiac changes [18]. The cardiotoxicity and nephrotoxicity, in addition to the intravenous use by slow infusion (3–4 h), prevent its use outside the hospital environment, which can be a problem for the patient and increases therapy costs.

Of all available drugs for leishmaniasis treatment, AmB is the most extensively studied in recent years in terms of the development of new treatments using DDS, as observed in Table 3. Liposomes are the main lipid systems studied in this respect. AmBisome, the first nanotechnological liposome approved for clinical use in the treatment of leishmaniasis, continues to be investigated in different models of infection and routes of administration [90, 91]. In this liposomal formulation AmB is strongly adsorbed to the lipids and therefore is less available to interact with the host cell membranes, therefore it generates fewer adverse effects and enables reduction in the number of doses required. A comparison with similar systems such as Fungisome, a liposome with larger vesicles marketed in India, indicates that the smaller size of the AmBisome liposomal vesicle could be related to increased blood circulation of the drug and accumulation in the lesion, explaining its greater effectiveness [92]. However, its high cost limits access to all patients, so there is still a huge demand for cheaper liposomal systems and for other lipid systems, such as nano and microemulsions [93–95]. In addition, AmBisome®

**Table 3** Amphotericin B – loaded DDS

| Amphotericin B | | | | | | | |
|---|---|---|---|---|---|---|---|
| Route | Nanosystem | Size (nm) | Parasite | Model | Regimen | Efficacy/outcome | Refs. |
| IP | Conventional liposome | 60–100 | *L. tropica* | BALB/c or C57BL/6 mice | 5 mg/kg every 3 days for a total of six doses | One day after treatment no significant decrease in parasite load was observed in C57BL/6 while BALB/c showed a modest response | [122] |
| IP | Apolipoprotein-stabilized phospholipid bilayer disk complexes (AmB-ND) × AmBisome® | 8–20 | *L. major* | BALB/c mice | 5 mg/kg at 24 h, 48 h, and 10, 20, 30, and 40 days post-infection | AmB-ND was 10- to 100-fold better in decreasing parasite load than AmBisome® | [123] |
| IP | PADRE-derivatized-dendrimer complexed with liposome (PDD/LAmB) | 148 | *L. major* | BALB/c mice | 6.25 mg/kg once a day for 10 days | 12 days after treatment mice treated with PDD-LAmB had a significantly lower ($P < 0.05$) parasite burden in the spleen than those treated with AmB at 37.5 mg/kg | [124] |
| IP | Dendrimer (AD) | ND | *L. major* | BALB/c mice | 50 mg/kg on alternate days for 42 days | After treatment with AD, just few parasites ($1 \pm 1$) were detected in spleen, liver, or footpad | [125] |
| IP | Anionic linear globular dendrimer (ALGD) | $138 \pm 11$ | *L. major* | BALB/c mice | 50 mg/kg on alternate days for 20 days | ALGD was as potent as the AmB, but it was safer | [126] |
| IP | Chitosan coated PluronicF127 micelles (Cs-PF-AmB-M) | $98 \pm 9$ | *L. donovani* | Syrian hamsters | 1 mg/kg for 5 consecutive days | One week after treatment Cs-PF-AmB-M inhibited 76% of parasite load in spleen compared to 60% for AmB | [127] |
| IP | Chitosan nanoparticles (AK) | ND | *L. major* | BALB/c mice | 10 mg/kg on alternate days for 42 days | After treatment with AK, no parasites were detected in spleen, liver, or footpad | [125] |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| IP | Peptide coated iron oxide nanoparticles (AmB-GINPs) | ≈10 | *L. donovani* | Hamsters | 5 mg/kg AmB for 5 consecutive days | 7 days after treatment AmB-GINPs was twofold more effective than AmB | [128] |
| IP | PLGA-DMSA nanoparticles (Nano-D-AmB) | 365 ± 71 | *L. amazonensis* | C57BL/6 mice | 6 mg/kg on the 1st, 4th and 7th days and 2 mg/kg on the 10th day | Nano-D-AmB promoted a significant ($P < 0.05$) reduction in parasite load compared with AMB (2 mg/kg for 10 days) | [129] |
| IV | Conventional liposome (AmBisome®) | 100 | *L. major* | BALB/c mice | 6.25, 12.5, 25, and 50 mg/kg once a day on six alternate days | AmBisome® between 12.5 and 50 mg/kg induced a lesion reduction while free drug was ineffective at nontoxic doses (<1 mg/kg). 7 weeks after treatment all cured mice had relapsed | [90] |
| IV | Conventional liposome (AmBisome®) | ND | *L. infantum* | BALB/c mice | 3 doses of 2 mg/kg with an interval of 5 days between the doses | 15 days after treatment AmBisome® induced a reduction in parasite log in the liver (3.5) and spleen (4.5) against (2.8 and 3.8, respectively) for free drug | [91] |
| IV | Conventional liposomes (DSHemsPC × AmBisome®) | 10 | *L. major* | BALB/c mice | 5 mg/kg on days 1, 2, 4, 7, 14, 21, and 28 post-infection | DSHemsPC and AmBisome® reduction in spleen parasite load was similar | [93] |
| IV | Conventional liposomes (Fungisome® – F × AmBisome® – A) | 220 | *L. major* | BALB/c mice | 5, 10, or 15 mg/kg on days 0, 2, 4, 6, and 8 | The $ED_{50}$ for parasite load was 4 for F and 3 for A. F was toxic at 15 mg/kg | [92] |
| IV | Chitosan and chondroitin sulfate nanoparticles (NQC-AmB) | 136 ± 11 | *L. amazonensis* | BALB/c mice | 1 mg/kg for 10 days | 30 days after treatment NQC-AmpB displayed better results in reducing the parasite load when compared to AmB | [130] |
| IV | Phos-anchored PLGA nanoparticle | 148 ± 9 | *L. donovani* | Hamsters | 1 mg/kg for 5 days | One week after treatment Phos-PLGA induced 82% of parasite | [131] |

(continued)

**Table 3** (continued)

| Amphotericin B | | | | | | | |
|---|---|---|---|---|---|---|---|
| Route | Nanosystem | Size (nm) | Parasite | Model | Regimen | Efficacy/outcome | Refs. |
| | | | | | | inhibition compared to 70% for AmB | |
| IV | MCT, Tween 80, cholesterol, and BHT nanoemulsion | 163 to 239 | *L. infantum* | BALB/c mice | 1 or 2 mg/kg for 5 alternative days | 2 mg/kg formulation led to significant reduction in parasite burden in the liver and spleen | [94] |
| IV | Miglyol® 812 and Lipoid® S100 microemulsion | 36 | *L. donovani* | BALB/c mice | 100 μL for 3 alternate days (1 mg/kg/day) | Reduction in parasite burden similar to that of AmBisome® in the same treatment regimen | [95] |
| SC | Poloxamer 407-micelles | ND | *L. amazonensis* | BALB/c mice | 25 μg in 100 μL for 15 days | One day after treatment Amp/M induced a reduction in parasite log in the lesion (3.3) against (4.9) for AmB | [132] |
| SC | Poloxamer P407 – micelles (Amp/M) | ND | *L. infantum* | BALB/c mice | 1 mg/kg for 15 days | 15 days after treatment Amp/M induced a reduction in parasite log in the liver (4.8) and spleen (5.8) against (2.8 and 3.8, respectively) for AmB | [91] |
| IL | Chitosan platelets | 689–1,000 | *L. major* | BALB/c mice | 100 μL each 2 days for 13 days | Decrease of the inflammatory granuloma and reduction of the parasitic load, in comparison with free drug | [133] |
| IL | Conventional liposome (AmBisome®) | 100 nm | *L. major* | BALB/c mice | 25 mg/kg once a day on six alternate days | No significant activity | [90] |
| IL | PLGA nanoparticle (AmB/PLGA) | 500–20,000 | *L. amazonensis* | BALB/c | 5 μg/10 μL of PBS (0.2 mg/kg) in a single dose | AmB/PLGA in single dose had more efficacy than the free drug in eight doses in early and established lesions, after 110 or | [102] |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | | 60 days after treatment, respectively | |
| IL | PLGA nanoparticles (AmB PLGA) | 90 | *L. major* | BALB/c mice | 1 mg/kg in a single dose | 34 days after treatment AmB NPs elicited a significantly lesion-reducing effect compared with free drug | [103] |
| Topical | Conventional liposome (SinaAmphoLeish 0.4%) | 80 | *L. mexicana* | BALB/c 129SVE mice | SinaAmphoLeish 0.4% gel on lesions twice every day for 10 weeks | No significant activity | [134] |
| Topical | Gamma-cyclodextrin | 1 | *L. amazonensis* | Hamster | 0.125% w/w for 21 days | No significant activity 5 weeks after treatment | [135] |
| Topical | Liposomes containing 0.1, 0.2, and 0.4% AmB | 100 | *L. major* | BALB/c mice | Twice a day for 4 weeks | Superiority of lip-AmB 0.4% compared to lip-AmB 0.2 and 0.1%. With 0.4% formulation, the parasite was completely cleared from the skin site of infection and spleens | [101] |
| Oral | GCPQ nanoparticles | 150 | *L. infantum* | BALB/c mice | 5 mg/kg for 5 or 10 days | Only after 10 days of treatment, AmB was able to reduce parasite load in liver (99%) and spleen (92%) as AmBisome® (5 mg/kg IP – single dose) | [98] |

(continued)

**Table 3** (continued)

| Amphotericin B | | | | | | | |
|---|---|---|---|---|---|---|---|
| Route | Nanosystem | Size (nm) | Parasite | Model | Regimen | Efficacy/outcome | Refs. |
| Oral | Nanosuspension | 528 | *L. donovani* | BALB/c mice | 5 mg/kg for 5 days | 2 days after treatment, AmB induced 29% of parasite inhibition in liver compared to untreated control | [99] |
| Oral | Carbon nanotubes | 40–70 (diameter) 2,000–8,000 (length) | *L. donovani* | Syrian golden hamster | 15 mg/kg for 5 days | Oral AmB had a similar effect to AmBisome® (5 mg/kg IP – single dose) inhibiting parasite burden in >95% | [100] |

*AmB* Amphotericin B, *DMSA* Dimercaptosuccinic acid, *DSHemsPC* 1,2-Distigmasterylhemi-succinoyl-sn-glycero-3-phosphocholine; $ED_{50}$–50% effective dose – mg/kg; GCPQ – N-palmitoyl-N-methyl-N,N-dimethyl-N,N,N-trimethyl-6-O-glycol chitosan; *IL* Intralesional route, *IP* Intraperitoneal route, *IV* Intravenous route, *PADRE* Pan-DR-binding epitope, *Phos* 3-O-sn-Phosphatidyl-L-serine, *PLA* Poly(D,L-Lactide, *PLGA* Poly(lactic-co-glycolic acid, *SC* Subcutaneous route, *ND* Not described

efficacy for CL has not yet been well established, its accumulation in the lesion after intravenous administration is related to local inflammation of the tissue, varying with the stage of the disease and the species of Leishmania [96]. While intralesional administration is known to have no effect [90].

Despite the recommendations of the DNDi and the WHO for the development of leishmaniasis treatments for oral and local administration and the vast amount of research already in this area, even today the intravenous and intraperitoneal routes are still the most studied in in vivo studies as can be observed in Table 3 [14, 97]. The oral polymeric nanosystems or CNTs loaded with AmB at 5–15 mg/kg administered for 5 or 10 days have been demonstrated to have good efficacy for VL treatment even when compared with a single dose of AmBisome at 5 mg/kg administered by the intraperitoneal route [98–100].

For local treatment of CL, the intralesional route has shown more promising results than the topical one. Topically, only a liposomal formulation containing 0.4% AmB was effective in a murine model of infection with *L. major*. However, in this study, no reference drug was used as a control and only 4% of the total drug from the formulation applied was able to permeate the mouse skin after 24 h, as assessed in a Franz diffusion cell assay [101].

The use of micro and nanoparticles of PLGA intralesionally in a single dose has emerged as an excellent alternative for local CL treatment. The treatment of mice infected with *L. amazonensis* using a single dose of AmB-loaded PLGA microparticles (0.2 mg/kg) showed excellent activity in both an initial and established lesion, leading to a reduction of 85% and 97% in the parasitic load, respectively, even 2 months after the end of treatment [102]. Meanwhile, the treatment of *L.* major-infected mice using a single dose of PLGA nanoparticles loaded with AmB (1 mg/kg) demonstrated a reduction in the lesion compared to the free drug 34 days after infection [103]. Unfortunately, the lack of data on parasitic load, the difference in models and doses used in these two studies does not allow us to establish a correlation between particle size and the effectiveness of the systems. However, these promising results, combined with well-defined safety profile, versatility to load different drugs, and controlled local release (fast and slow) of drugs, due to the presence of PLGA particles with different sizes, show the potential of these DDS systems for the local treatment of CL with a single dose [104, 105].

## *2.3 Pentamidine*

Considered a second-line drug for leishmaniasis, pentamidine is an aromatic diamidine-based compound with efficacy comparable to the antimonials but less active than AmB. It is a repurposed drug, developed initially as an insulin analogue [17]. The drug acts mainly on the parasite mitochondrion and nucleus by affecting enzymes such as mitochondrial topoisomerase, S-adenosyl-L-methionine decarboxylase, and nucleoside triphosphate diphosphohydrolase (NTPDase) 1 [106]. The drug is administered by the intramuscular route or by intravenous infusion, with

**Table 4** Pentamidine-loaded DDS

| Pentamidine | | | | | | | |
|---|---|---|---|---|---|---|---|
| Route | Nanosystem | Size (nm) | Parasite | Model | Regimen | Efficacy/ outcome | Refs. |
| IV | Methacrylate nanoparticles | 270 to 330 | *L. major* | BALB/c mice | 0.17 mg/kg/day on days 13, 15, and 17 after infection | 21 days after infection, the treatment with nanoparticles caused a reduction in amastigotes of 77% in comparison with free drug | [108] |
| IV | PLGA nanoparticles | 150 ± 20 | *L. infantum* | BALB/c mice | 0.055, 0.11, 0.22 or 0.44 mg/kg on days 14, 16, and 18 after infection | After 21 days of infection, treatment with PLGA-Pent was 3.3 times more effective than free drug | [110] |
| IV | Methacrylate nanoparticles | ND | *L. infantum* | BALB/c mice | 0.05, 0.09, 0.17 and 0.24 mg/kg on days 14, 16 and 18 after infection | After 21 days after infection, pentamidine-loaded nanoparticles at 0.24 mg/kg showed similar parasite load suppression to free drug, but in a dose 10 times lower | [109] |

*Pent* Pentamidine, *PLGA* Poly(lactic-co-glycolic acid, *IV* intravenous route, *ND* Not described

many related side effects including pain at the injection site, vomiting, headache, hypotension, syncope, nephrotoxicity, diabetes, transient hyperglycemia, and hypoglycemia [18].

Pentamidine is no longer widely used for VL treatment due to low rate of cure and arising cases of resistance in endemic areas [107], but it is still considered an option for CL [12]. The drawbacks related to pentamidine have weakened the interest of research groups in developing formulations to improve the therapeutic efficacy of this drug. As can be observed in Table 4 only three publications were found for DDS encapsulating pentamidine, and all of them employed PLGA or methacrylate

nanoparticles to improve drug efficacy in vivo [108–110]. In a CL model with *L. major*, pentamidine in methacrylate nanoparticles given intravenously at 0.17 mg/kg/day on days 13, 15, and 17 post-infection was able to efficiently reduce the number of amastigotes at the lesion site compared to the free drug [106]. In VL infection caused by *L. infantum*, three doses of PLGA or methacrylate nanoparticles containing pentamidine by the intravenous route on days 14, 16, and 18 post-infection significantly suppressed parasite load in contrast to when the free drug was used [108–110].

Taking into account all the innovations regarding DDS development, it is perhaps the right time to rethink the use of pentamidine encapsulated in DDS for leishmaniasis treatment. Formulations for local treatment or for macrophage targeting will reduce the required effective dose and consequently the adverse effects related to excess drug in the body [24].

## *2.4 Miltefosine*

Miltefosine is the unique oral drug approved for leishmaniasis treatment. It is a hexadecylphosphocholine initially developed to treat breast cancer and repositioned to treat VL in South Asia [23]. Its mode of action is still controversial, with some suggesting the effects are attributed to modulation of phospholipid biosynthesis [111], induction of apoptosis-like death [112], and intracellular calcium imbalance [113]. Clinical studies in patients infected with species predominant in Americas revealed variable efficacy among these *Leishmania* species [16] meaning its clinical use is not approved in the American continent. As miltefosine has a long elimination half-life, around 152 h, its accumulation in the body may increase toxic effects, such as gastrointestinal complications and teratogenicity, which hinders its use in women of childbearing age [18].

As observed for pentamidine, few reports have been found for DDS and miltefosine. Despite the current oral administration route and the DNDi guidelines for new treatments for leishmaniasis [14], all three proposed formulations aim at an invasive treatment, intraperitoneal or intralesional [83, 114, 115]. Tripathi and colleagues have reported the effectiveness of a hybrid formulation consisting of chitosan nanostructured lipid carriers stabilized by miltefosine and entrapping AmB (HePC-AmB-NLC) [114]. In this study, HePC-AmB-NLC was tested against *L. donovani* in a VL model using golden hamsters at 1 mg/kg/day of AmB administered intraperitoneally for 5 consecutive days. The treatment caused parasite inhibition of 85% in comparison with 68% for the control group using a formulation without miltefosine (Tween 80-AmB-NLC) [114]. Another work reported the application of PLGA–PEG nanoparticles carrying miltefosine (PPEM) for the treatment of *L. donovani*-infected hamsters. PPEM delivered intraperitoneally at 2.5 mg/kg for 5 days inhibited 94% of the parasite load in the spleen, whereas the free drug caused 75% of inhibition [115]. A liposomal formulation with miltefosine was also reported for an intralesional treatment of BALB/c mice infected with *L. major*. In this work,

Momeni et al. showed that among different DDS formulations only miltefosine-loaded liposomes caused a significant reduction in the parasite load in comparison with an untreated group [83]. Besides the administration route, the encapsulation of miltefosine in DDS did not result in significant improvements in the therapeutic efficacy of the drug, as observed in Table 5.

## *2.5 Paromomycin*

Paromomycin (PM) is an aminoglycoside-aminocyclitol antibiotic employed for VL treatment as a parenteral formulation and by the topical and oral route for CL treatment. The drug acts by altering protein biosynthesis, due to an interaction with the parasite 30S ribosomal subunit impairing the initiation of protein synthesis and misreading of mRNA template [116]. Through the parenteral route, at 15 mg/kg, the free drug showed variable efficacy against VL. Whilst topical formulations of paromomycin at 15% plus 12% methylbenzethonium chloride or 10% urea exhibited good effectiveness against Old World *Leishmania* species (*L. major*) but efficacy was lower with New World species (*L. panamensis* and *L. braziliensis*) [12, 18]. The most common side effects are pain at injection site, nephrotoxicity, ototoxicity, and increase of hepatic transaminases [18].

The drug has a low distribution volume after intramuscular injection, with higher concentrations found in renal cortex and in the inner ear, which explain its nephron and ototoxicity [116]. Additionally, poor oral absorption and fast elimination half-life averaged between 2 and 3 h compromise the drug bioavailability [116].

Lipid formulations were the principal DDS chosen for encapsulating paromomycin with in vivo results (Table 6), fluctuating between liposomes and solid lipid nanoparticles. Solid lipid nanoparticles encapsulating paromomycin were tested against *L. major* and *L. tropica* in vivo in BALB/c mice by the intramuscular and/or intralesional routes [117, 118], in both cases the formulation reduced parasite load in comparison with the free drug, showing an increase in efficacy and drug bioavailability due to DDS encapsulation. Liposomal paromomycin formulations were proposed to increase the drug efficacy by the topical route. In Table 6, it can be noted that the encapsulation of paromomycin in transfersome or liposomes increased significantly the drug efficacy topically in a CL model caused by *L. major* [119–121]. Interestingly, the paromomycin encapsulation in mannosylated thiolated chitosan PLGA nanoparticles was able to enhance macrophage uptake and parasite killing in vitro, as well as efficacy by the oral route in a murine VL model [57]. It opens new perspectives for the use of paromomycin by a non-invasive route for VL treatment.

Overall, the encapsulation of paromomycin in all proposed formulations resulted in satisfactory parasite load reduction in murine models of CL and VL. There is one report in the literature on a clinic trial employing a liposome-paromomycin formulation for CL topical treatment.

**Table 5** Miltefosine-loaded DDS

| Miltefosine | | | | | | | |
|---|---|---|---|---|---|---|---|
| Route | Nanosystem | Size (nm) | Parasite | Model | Regimen | Efficacy/outcome | Refs. |
| IP | Miltefosine-AmB-nanostructured lipid carrier (HePC-AmB-NLC) | 151 ± 8 | *L. donovani* | Golden Hamsters | 1 mg/kg/day AmB (IP) for 5 consecutive days | 15 days after treatment, HePC-AmB-NLCs caused 85% of parasite inhibition against control group 68% (Tween 80-AmB-NLC) | [114] |
| IP | PLGA–PEG nanoparticles (PPEM) | 15 ± 5 | *L. donovani* | Hamsters | 2.5 mg/kg for 5 consecutive days | After 24 days of infection PPEM caused 94% of parasite inhibition. While free drug induced 75% of inhibition in the spleen | [115] |
| IL | Liposome | 166 ± 20 | *L. major* | BALB/c mice | 293 μg/mL 2× week for 35 days | 35 days after treatment liposomal miltefosine induced parasite load inhibition ($P > 0.05$) in comparison with untreated group | [83] |

*PEG* Poly(ethylene glycol), *PLGA* Poly(lactic-co-glycolic acid), *AmB* Amphotericin B, *IP* Intraperitoneal route, *IL* Intralesional route

**Table 6** Paromomycin-loaded DDS

| Paromomycin | | | | | | | |
|---|---|---|---|---|---|---|---|
| Route | Nanosystem | Size (nm) | Parasite | Model | Regimen | Efficacy/outcome | Refs. |
| IM | Solid lipid nanoparticle PM-SLN | 120 | *L. major* | BALB/c mice | 30 or 50 mg/kg 2×/week for 4 weeks | Eight weeks after infection, treatment with PM-SLN (30 or 50 mg/kg IM) reduced parasite burden ($P < 0.001$) in comparison with free drug | [117] |
| IM or IL | Solid lipid nanoparticle PM-SLN | 120 | *L. tropica* | BALB/c mice | 15, 30, or 50 mg/kg 2×/week for 4 weeks | Ten weeks after infection, treatment with PM-SLN (50 mg/kg IM) or (15 mg/kg IL) reduced parasite burden ($P < 0.001$) in comparison with free drug | [118] |
| Topical | Transfersome (PMTF) | 200 | *L. major* | BALB/c mice | 50 mg of formulation with 10% PM 2×/day for 4 weeks | 4 weeks after treatment, animals treated with PMTFs showed a significantly lower parasite burden than groups received PM cream ($P < 0.001$, spleen) | [119] |
| Topical | Liposome | 269 ± 88 | *L. major* | BALB/c mice | 50 μL of liposome with 5% PM 2×/day for 12 days | 100 days after treatment, mice treated with liposome showed 30% of cure rate in comparison with 0% of those treated with free drug | [121] |
| Topical | Liposome Lip-PM | 532 ± 164 (10%) 508 ± 250 (15%) | *L. major* | BALB/c mice | 50 mg of formulation with 10 or 15% PM 2×/day for 4 weeks | 4 weeks after treatment, lip-PM formulation, a complete cure of the lesions was observed and significantly lower parasite burdens ($P < 0.001$) in spleen, compared to PBS or empty liposome | [120] |
| Oral | Mannosylated thiolated chitosan PLGA nanoparticles | 391 ± 7 | *L. donovani* | BALB/c mice | 20 mg/kg/day for 1 week | 14 days after treatment, MTC-PM-PLGA caused 3.6-fold reduction of parasitic burden in liver in relation to free drug | [57] |

*PM* Paromomycin, *SLN* Solid lipid nanocapsules, *PLGA* Poly(lactic-co-glycolic acid), *IL* Intralesional route, *IM* Intramuscular route

## 3 Conclusion

DDS have several benefits over the classical chemotherapy of leishmaniasis thus enhancing drug efficacy, reducing toxicity, and allowing alternative administration routes for both current and new drugs. Among the DDS utilized for current antileishmanials covered in this review, lipid systems are the most studied, likely due to their greater biocompatibility and the pioneering clinical use with liposomal amphotericin B that proved to be effective. With regard to the drug, amphotericin B is the most studied followed by pentavalent antimonials, especially meglumine antimoniate, probably because of their potent antiparasitic activities, and long and broad uses as first-line drugs, respectively. The most explored animal model is the BALB/c mouse both for CL and VL, but hamsters are often used for VL caused by *L. donovani*. In terms of administration route, DDS are mostly reported for intravenous and intraperitoneal injections. Despite all these studies and the different combinations that have already been achieved, with the exception of a few studies using liposomal formulations with amphotericin B, there are currently no other clinical trials on DDS for leishmaniasis (clinicaltrials.gov). Although DDS can improve drug permeation through mucosa and skin and encourage the novel use of "old" drugs through the preferred oral and topical routes, consistent stability and scale-up studies are also necessary to impact on leishmaniasis treatment.

**Compliance with Ethical Standards** *Conflict of Interest*: The authors declare that they have no conflict of interest.

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# Pharmacological Treatment of Malaria


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## Contents



## Abstract

- Chloroquine is currently used as a monotherapy in regions with chloroquine-susceptible infections. In contrast, the drug is no longer recommended for the prophylaxis against *P. falciparum*.
- ACT is nowadays the reference treatment option recommended by the WHO because of its efficacy compared to non-artemisinin combination therapy.
- Lumefantrine has never been used as a monotherapy in the treatment of malaria. However, its combination with artemether represents the most effective ACT recommended by the WHO for the treatment of uncomplicated *P. falciparum* malaria.
- Mefloquine monotherapy is largely used for the prevention of malaria in all areas where no resistance to antimalarial drugs is recorded.

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- Mefloquine-artesunate ACT is well tolerated and associated with mild side effects, and it is thus recommended by the WHO for the treatment of uncomplicated *P. falciparum* malaria.
- Quinine is the preferred antimalarial drug for pregnant women and the treatment of multidrug-resistant malaria. In combination with antibiotics, quinine has improved antimalarial efficacy and decreased side effects, and it is nowadays considered as a second-line option that is an alternative to ACTs when the latter is not available.

**Keywords** Antimalarials, Artemisinin-based combination therapy, Chloroquine, Malaria, Primaquine

## 1 Introduction

Malaria is a ubiquitous hematoprotozoan parasitic disease that is due to infection by protozoa belonging to the genus *Plasmodium*, which includes five species: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae,* and *P. knowlesi* [1, 2]. These parasites are transmitted to the hosts through the bite of the female Anopheles mosquito and infect mammals, birds, and reptiles, which suggests an early origin. The first evidence of malaria parasite was detected in a mosquito from the Paleogene period (around 30 million years ago). Description of symptoms that are compatible with malaria disease is found in documents from ancient Egyptians, dating back to around 1,550 B.C. and ancient Greeks (fourth century B.C.), particularly describing the association between intermittent fever and wet ground or climatic and environmental conditions [3, 4]. Subsequently, in the renaissance period malaria spread in Africa and Europe with heavy outbreaks in Italy and England. In this period, the use of medicinal plants to treat malaria symptoms raised, and several herbal medications were used to relieve the pain in infected patients. The diffusion of malaria to the Americas was probably related to the Atlantic slaves trade in the sixteenth century, although several African slaves developed resistance to malaria. In more modern eras, malaria spread globally with the only exception of Antarctica. The exploitation of medicinal plants such as *Cinchona officinalis* [5] and *Artemisia annua* [6] as well as the isolation and synthesis of active principles paved the way to the current therapeutic options, which are the focus of this chapter.

According to the World Malaria Report 2020 from the World Health Organization (WHO), in 2019 there was an estimate of 229 million cases of malaria in 87 endemic countries, while 29 of them account for 95% of malaria cases, with the largest part being in Africa (94% of global cases). Notably, five countries including Nigeria (27%), the Democratic Republic of the Congo (12%), Uganda (5%), Mozambique (4%), and Niger (3%) accounted for about 51% of all cases globally. Although there is a significant trend in the reduction of malaria mortality in the last 20 years (409,000 deaths in 2019 vs. 736,000 deaths in 2000), a slight

increase of infections and total deaths was observed compared to 2018 (380,000 deaths) [7].

More than 24 million children were estimated to be infected in sub-Saharan Africa, showing prevalence of severe or moderate anemia compared to non-positive children. Moreover, about 12 million pregnant women were exposed to malaria infection, who delivered about 822,000 children with low birth weight [7].

## *1.1 Life Cycle of Malaria Parasites*

In the case of human infection, the life cycle of the malaria parasite involves two hosts, namely humans and infected mosquitos, and it has two stages in humans (Fig. 1) [8].

In the first stage, which is commonly referred to as the pre-erythrocytic stage, the *Plasmodium*-infected female Anopheles mosquito inoculates plasmodial sporozoites that are maturated in the mosquito's salivary glands into the human host skin during a blood meal. Usually, less than ten sporozoites are inoculated into the human host by an infected mosquito. Sporozoites enter into the liver due to their ability to

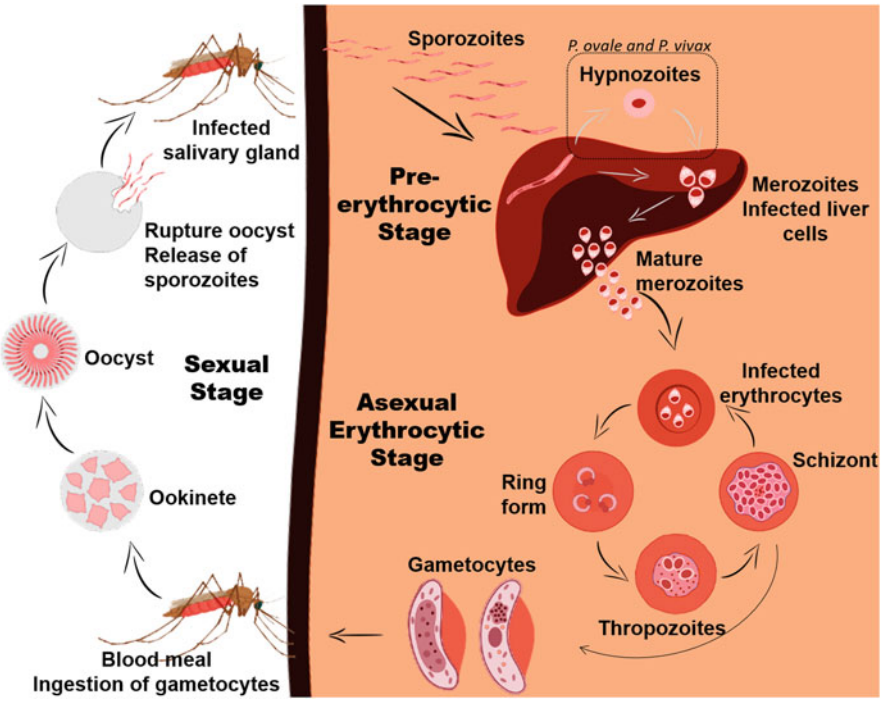


**Fig. 1** Schematic representation of the *P. falciparum* parasite life cycle, highlighting the dormant condition found in *P. ovale* and *P. vivax*

traverse through host cells to reach the hepatocytes [9]. The parasite develops into tissue schizonts which, after rupture, can release thousands of blood-infective merozoites to the bloodstream to begin the 48-h asexual reproduction cycle. *P. vivax* and *P. ovale* can remain as hypnozoites (dormant condition) in the liver and are responsible for malaria relapse. This stage is symptomatically silent [10].

The second stage is also referred to as asexual blood-stage infection, and it starts with the release and asexual multiplication of the merozoites in the erythrocytes, which develop into immature ring stage trophozoites – asexual erythrocytic stage. Once in the erythrocytes, the parasite modifies the host red blood cell, and several nuclear division cycles take place until the parasite consumes most of the content of the red cells. The mature schizonts burst and release merozoites into the bloodstream, and each merozoite repeats the asexual life cycle every 24, 48, or 72 h depending on the species. In this stage, malaria patients show clinical signs of illness and complications that are typical of the disease [11]. All the symptoms can be associated with asexual erythrocytic or blood-stage parasites and include diarrhea, fever, headache, weakness, vomiting, cough, and abdominal pain. If drugs are readily available, mortality due to *P. falciparum* malaria is about 0.1%. In contrast, if the parasite replicates itself without any pharmacological interference, more severe symptoms appear as vital-organ dysfunction, acidosis, and anemia, and mortality rises to about 20% [12].

Some merozoites discriminate into sexual erythrocytic stages and develop into immature gametocytes, which can be ingested by a non-infected Anopheles mosquito, thus initiating the sexual stage and development. The gametocytes differentiate into male and female gametes which fuse together for generating zygotes. The ookinetes are formed from zygotes and penetrate the mosquito midgut wall allowing the development of the oocysts. The sporozoites developed from the matured oocysts migrate into the mosquito's salivary glands and await inoculation into the next host, starting a new cycle and promoting the pathogen's spread among individuals [13].

The malaria eradication era initiated in the late 1940s, thanks to regional actions. These were followed by the Global Malaria Eradication Program launched by the WHO in 1955. These programs aimed at the elimination of malaria parasites from endemic or affected countries, thanks to the application of drug development, vector control, and insecticides [14]. Although the successful eradication of malaria was achieved in Europe, North America, and parts of Asia and South-Central America, substantial failures were recorded in the sub-Saharan countries. Moreover, in some of the areas where malaria was eliminated (for example, in South America [7]) it appeared again due to the development of resistance to available first-line antimalarial treatments as well as technical difficulties in the execution and maintenance of eradication strategies, particularly in Africa. These challenges led to abandon the eradication programs in 1962 [15]. Thanks to new knowledge acquired in drugs and vaccines against malaria, and especially to a better understanding of the social, economic, and cultural dimensions of malaria, in 1992 new malaria eradication initiatives were launched and supported by a number of public and private organizations including – but not limited to – the WHO, Malaria No More [16], the Bill &

Melinda Gates Foundation [17], the Carter Center [18], and Medicines for Malaria Venture [19].

## 2 Drugs Against Malaria

At the state of the art, it is clear that eradication of malaria in Africa is not achievable with current tools, and that improved surveillance and a better understanding of transmission, environment, climate, and migration phenomena coupled with efficient transnational cooperation will be crucial to achieve the foreseen objectives. In addition, it is also evident that the current arsenal against malaria is not efficient enough to eradicate the disease, and that new antimalarial drugs endowed with novel/innovative mechanisms of action are urgently needed [20].

Several synthetic and natural products have been discovered to treat malaria in infected patients or to prevent the onset of the disease, but their adverse effects or the emergence of drug resistance have notably hindered their development and broad application [21, 22]. The complexity of the malaria parasite life cycle also limits the development of an efficient vaccine.

Most antimalarial drugs target the erythrocytic stage and not the hepatic stage, as the latest lacks clear symptoms being difficult to diagnose the disease at this stage. Moreover, most antimalarial drugs lost efficacy with time due to *P. falciparum* resistance. The ideal antimalarial agent should be active against the blood-stage, transversal to all drug-resistant parasites [23], devoid of cytotoxicity or genotoxicity, and efficient – preferably – with a once-daily oral administration. It is also highly important that the new drug is cheap, according to the Medicines for Malaria Venture. Unfortunately, most antimalarial drugs available to date fail to meet these requirements, and new drug development strategies have to be rethought accordingly [21].

A number of drugs able to impair the replication of *Plasmodium* spp. into the host are available and are characterized by different mechanisms of action and pharmacokinetics/toxicity profiles. Given the negative impact of drug resistance and the spread of drug-resistant strains of *Plasmodium* spp., two aspects should be carefully considered:

1. Administration of antimalarial drugs should be granted to confirmed cases of malaria, and adherence to the treatment protocol should be promoted, i.e. robust, low-cost, and rapid diagnostic tests able to discriminate between generic febrile illnesses and malaria, as well as health systems and sensitization campaigns that encourage patient's adherence to the therapy.
2. To avoid, or at least to delay, the insurgence of resistance towards a specific drug, the combination of at least two antimalarial drugs with different mechanisms of action should be administrated (combination therapy).

In the next sub-chapters, we summarize the main antimalarial individual drugs and combination therapies, with a focus on the chemical structures of the compounds, toxicological profiles, and insights into the mechanism of action.

## 2.1 Chloroquine

Chloroquine (Fig. 2) was first synthesized in Germany. Just in the 1940s, during World War II, the US military recognized its antimalarial activity and the drug was widely used for this purpose. From a mechanistic standpoint, chloroquine accumulates in the food vacuole of parasites, where it impairs the heme detoxification process and inhibits the synthesis of nucleic acids. Unfortunately, resistance to chloroquine was noted in Cambodia and Columbia already in the late 1950s, spreading all over the world and reaching Africa in 1978 [24]. *P. falciparum* resistance to chloroquine is the result of mutations in the *pfcrt* gene located on chromosome 7, which encodes a 48.6 kDa vacuole membrane transporter protein (*Pf*CRT) of 424 amino acids. In drug-resistant parasites, the *pcfrt* gene encodes for the Lys76Thr mutation. A second transporter, *Pf*MDR1, can also modulate the level of resistance in vitro. *Pfmdr*1 gene is located on chromosome 5 and encodes P-glycoprotein homologue 1. In this transporter, the substitution of aspartic acid to tyrosine in codon 86 is another mutation responsible for chloroquine resistance. Other polymorphisms such as Phe184, Cys1034, Asp1042, and Tyr1246 are also involved in chloroquine resistance [24, 25]. The wide diffusion of chloroquine-resistant malaria parasites triggered the discovery and development of new antimalarial drugs, most of which have been described above.

Chloroquine is currently used as a monotherapy in regions with chloroquine-susceptible infections either for the prevention or treatment of uncomplicated and severe malaria, especially due to *P. vivax*. In contrast, the drug is no longer recommended for the prophylaxis against *P. falciparum*.

At therapeutic doses used in the prophylaxis and therapy of malaria, chloroquine is generally well tolerated. Common side effects include pruritus, headache, liver toxicity, and gastrointestinal issues. More rarely, central nervous system toxicity is observed. It is worth noting that chloroquine and its metabolite hydroxychloroquine have been recently tested as an option for the therapy of coronavirus disease 2019 (COVID-19). Despite controversial and generally non-satisfactorily outcomes of

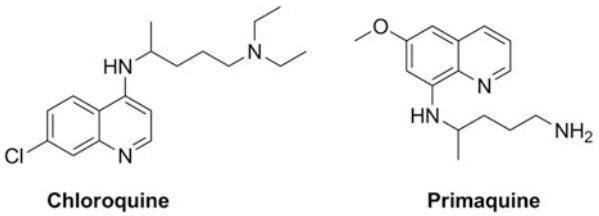


**Fig. 2** Chemical structure of chloroquine and primaquine

clinical trials, the extensive and prolonged use of chloroquine has highlighted additional adverse effects, particularly heart rhythm problems, liver or kidney injury, retinopathy, and hypoglycemia [26].

## 2.2 Primaquine

Primaquine (Fig. 2) is an 8-aminoquinoline derivative used in the treatment and prevention of relapse of *P. vivax* and *P. ovale* malaria, as well as in the reduction of *P. falciparum* transmission in areas where resistance to artemisinin derivatives is found. In addition, primaquine is used as a combination therapy in conjunction with artesunate, ACT, or chloroquine when no first-line alternatives are available, as well as an alternative option in malaria prophylaxis. The mechanism of action of primaquine is still not clear, but several studies suggest that it acts mostly in the exoerythrocytic hypnozoite and the sexual gametocyte stages of parasites, while it has weaker activity against the asexual stages of *P. vivax*, and no activity against *P. falciparum*. While primaquine itself is inactive at the molecular level, its metabolites produced by liver enzymes interfere with mitochondrial processes and electron transport in the parasite [27, 28].

The most common adverse effects include gastrointestinal disorders, which decrease by administration with food [29, 30], while rare effects include hypertension and arrhythmia.

## 2.3 Artemisinin-Based Combination Therapy (ACT)

Artemisinin-based combination therapies are effective combinations of antimalarial drugs, showing less than 5% treatment failure in many trials and multiple settings. The ACT is nowadays the reference treatment option recommended by the WHO because of its efficacy compared to non-artemisinin combination therapy [31, 32], even though it is generally associated with a higher cost than former drugs, which are no longer effective. Indeed, the ACT has several advantages as a fast reduction of the parasite growth, rapid clearance of symptoms, effectiveness against multidrug-resistant *P. falciparum*, blockage of transmission of gametocytes, and delay in the development of resistance [33]. The ACT consists of the combination of a rapidly acting artemisinin derivative with a long-acting antimalarial drug. The combination is designed to provide a rapid and massive clearance of the parasite by the artemisinin component followed by eradication and protection from the development of resistance to the artemisinin derivative by the longer-acting drug. This latter also guarantees a prophylaxis period after the treatment. At the state of the art, the WHO recommends five ACTs for the treatment of *P. falciparum* malaria:

1. artemether-lumefantrine;
2. artesunate-amodiaquine;
3. artesunate-mefloquine;
4. artesunate-sulfadoxine/pyrimethamine (SP);
5. dihydroartemisinin-piperaquine,

with (1) being the most effective. Individual drugs and their mechanism of action are discussed below.

### 2.3.1 Dihydroartemisinin, Artemether, and Artesunate

Artemisinin (Fig. 3) is a sesquiterpene lactone bearing a 1,2,4-tiroxane ring isolated from *Artemisia annua*. Since its discovery in 1972 by Youyou Tu, who received the Nobel Prize in Medicine in 2015, it is well known for its antimalarial properties [34, 35]. According to the proposed mechanism of action, the endoperoxide moiety of artemisinin and its derivatives interacts with the free iron ions of ferriprotoporphyrin (heme) in parasite food vacuoles, generating free radicals that are cytotoxic for the parasite. However, artemisinin itself has several drawbacks including low water solubility, bioavailability, short half-life, and neurotoxicity which limit its use in clinical practice, particularly as monotherapy. In addition, malaria parasites developed resistance to this natural product [36], preferentially within the Tyr86 allele of the *pfmdr1* gene, even though Ser1034, Asn1042, and Asp1246 alleles are also associated with artemisinin resistance [37]. Several approaches have been undertaken to optimize artemisinin, which led to the development of suitable drugs currently used in the antimalarial combination therapy [38, 39].

Dihydroartemisinin (Fig. 3) is the bioactive form of artemisinin and its derivatives, and it is used as a drug. Different from artemisinin, and similarly to the derivatives described in this section, dihydroartemisinin is not available in nature and is prepared through semi-synthetic transformations of artemisinin. Notably, dihydroartemisinin is also a valuable starting point for the preparation of other artemisinin derivatives including artemether and artesunate (Fig. 3). Despite the

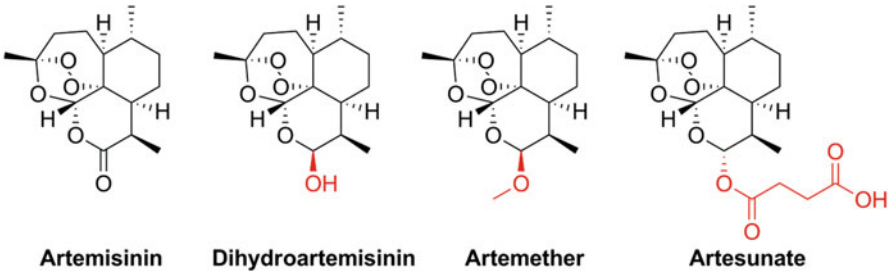


**Fig. 3** Chemical structure of artemisinin and its derivative currently used in ACTs. Chemical modifications to the natural artemisinin are highlighted in red

initial enthusiasm with this drug, several studies highlighted the chemical instability of dihydroartemisinin, as well as the technical challenges in the preparation of suitable and stable pharmaceutical formulations [40]. Compared to artemisinin, the labile stereocenter at C-10 originates two lactol hemiacetal epimers (i.e., α and β) that interconvert in solution through the opening of the lactol ring system. While at the solid-state dihydroartemisinin exists exclusively in the β-epimer form, its dissolution originates a mixture of α- and β-epimers with solvent-dependent composition [41]. An in vivo study on healthy volunteers and malaria patients showed dihydroartemisinin is mostly bound to serum proteins (93%) than in *Plasmodium*-infected individuals, and it exists preferably in the α-epimer form [42].

Artemether (Fig. 3) is an oil-soluble methyl ether derivative at position C-10 of dihydroartemisinin, which is then slowly converted to the parent bioactive derivative by the host's hepatic enzymes CYP3A4/5 [43]. The drug was discovered in 1987 in China through rational modifications of artemisinin within the framework of the well-known Chinese National Project 523 [44]. Chemical modification of artemisinin was indeed made possible by the challenging understanding of its chemical structure [45]. The same drug design strategy led to additional artemisinin derivatives such as dihydroartemisinin (discussed above) and the water-soluble artesunate (Fig. 3), which corresponds to the hemisuccinate ester of dihydroartemisinin. Artesunate is more soluble in water than other artemisinin derivatives, and its rapid conversion into the bioactive form is operated by host plasma esterase enzymes. Notably, intravenous or intramuscular artesunate monotherapy is used for at least 24 h in the treatment of severe malaria, until the patient can tolerate oral medications. Then, the therapy should continue with ACT.

Overall, these drugs are generally well tolerated at therapeutic doses, with mild adverse effects that include nausea, vomiting, cardiac dysfunctions, and in rare cases kidney failure and allergic reactions [46].

### 2.3.2 Lumefantrine

Together with some artemisinin derivatives, lumefantrine (Fig. 4) was discovered by the Chinese project 523 in 1976. Lumefantrine is a fluorene amino alcohol derivative that acts through a yet unelucidated mechanism of action. The most accepted hypothesis suggests that the drug prevents the synthesis of nucleic acids and proteins in the *Plasmodium* by complexation with hemin and the consequent inhibition of β-hematin formation. This mechanism is common to chloroquine (see Sect. 2.1) with which lumefantrine shares some key pharmacophoric and chemical features, even though it has a significant activity against chloroquine-resistant strains of *P. falciparum* [47]. From a chemical standpoint, lumefantrine is a racemic mixture of the two enantiomers, which show different pharmacokinetics features, such as underlined in a recent study, favoring the (+)-lumefantrine isomer [48].

Lumefantrine has never been used as a monotherapy in the treatment of malaria. However, its combination with artemether represents the most effective ACT recommended by the WHO for the treatment of uncomplicated *P. falciparum*

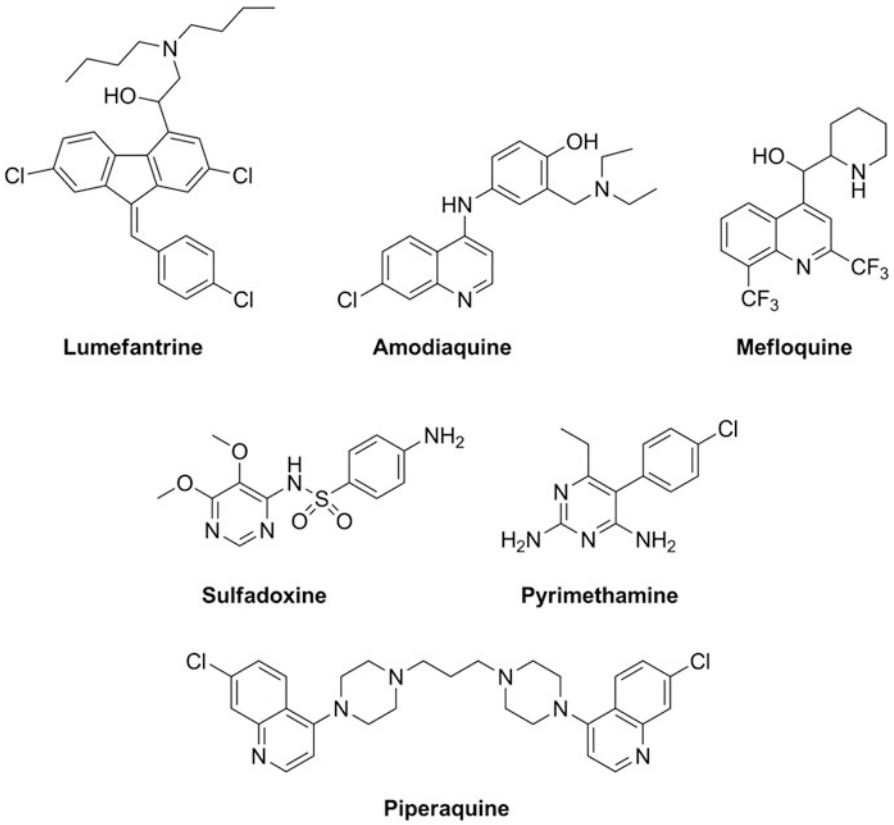


**Fig. 4** Chemical structure of synthetic antimalarial drugs used in ACTs

malaria [49]. This ACT, also referred to as co-artemether (or coartemether), is available in tablets for oral administration containing both artemether and lumefantrine at a 1:6 ratio. The ACT should be ideally administered twice a day for 3 days preferably after food because fats are reported to enhance the adsorption of lumefantrine. The drug is available also for children, while side effects are generally negligible (e.g., headache, sleep disorders, tinnitus) up to allergic reactions.

### 2.3.3 Amodiaquine

Amodiaquine (Fig. 4) is a 4-aminoquinoline derivative with higher potency and faster recovery time than the reference antimalarial chloroquine, an effect that is related to its inherent antimalarial and antipyretic activity. Therefore, amodiaquine became a very efficient and low-cost alternative to chloroquine [50]. Amodiaquine shares chemical and pharmacophoric features with chloroquine, as well as a similar mechanism of action. The bioactive metabolite desethylamodiaquine is generated by

CYP3A4 and CYP2C8 enzymes, and it is thought to accumulate in food vacuoles of the parasite where it interferes with heme detoxification. Nevertheless, amodiaquine is active against *Plasmodium* strains that acquired resistance to chloroquine [49]. While amodiaquine monotherapy failed to treat malaria patients, particularly underweighted children, the WHO recommends its use in combination with artesunate. The ACT is available as fixed-dose tablets with artesunate:amodiaquine ratio as 1:2.7 to be administered once a day for 3 days. The drug combination is generally well tolerated, with side effects on the gastrointestinal tract such as nausea and pains [51, 52]. Other common side effects include anorexia, cough, and weakness whereas more severe adverse effects are rare and are associated with prolonged prophylactic use of the drug [53].

### 2.3.4 Mefloquine

Fluoroquinolones are broad-spectrum antibacterial compounds that are structurally related to quinine, a natural product extracted from *Cinchona officinalis* and formerly recommended as a first-line treatment of malaria [54]. Their application in the treatment and prophylaxis of malaria is restricted to mefloquine (Fig. 4), which offers stronger potency and fewer side effects than the parent quinine.

From a chemical standpoint, mefloquine exists in two racemic forms, with the *erythro* enantiomers being effective against malaria. However, the pharmacological profile of *erythro* enantiomers is different, although their mixture represents the composition of one of the most important antimalarial drugs, i.e. Lariam. Several studies focused on the determination of the absolute configuration of both enantiomers, showing that (+)-*erythro*-mefloquine with absolute configuration 11S,12R is responsible for the antimalarial activity, whereas the (−)-*erythro* form is considered the major determinant for the adverse effect of the drug, also providing a limited antimalarial activity in vitro and in vivo [55–57].

The mechanism of action of mefloquine is not fully elucidated. However, in analogy to quinolone antimalarials, mefloquine is suggested to inhibit parasite-mediated heme detoxification [54]. Recently, the capability of mefloquine to inhibit parasite-mediated endocytosis of the cytosol has also been proposed [58].

Mefloquine monotherapy is largely used for the prevention of malaria in all areas where no resistance to antimalarial drugs is recorded. To this aim, the drug is taken orally for 1 or 2 weeks before entering the area where malaria is endemic, or there is a concrete risk of infection by *Plasmodium* spp. However, mefloquine alone is associated with several adverse effects, particularly at the central nervous system level [59].

In contrast, the mefloquine-artesunate ACT is well tolerated and associated with mild side effects, and it is thus recommended by the WHO for the treatment of uncomplicated *P. falciparum* malaria. The ACT is formulated in fixed-dose tablets for pediatric and adult use, containing artesunate and mefloquine in ratio 1:2.2 to be administered once a day for 3 days.

Cases of resistance to mefloquine have been recorded in Asia, highlighting Ser1034, Asn1042, and Asp1246 mutations in the *pfmdr1* gene as the cause of drug resistance [60].

### 2.3.5 Sulfadoxine/Pyrimethamine (SP)

Sulfadoxine inhibits dihydropteroate synthase (DHPS) while pyrimethamine inhibits dihydrofolate reductase (DHFR) (Fig. 3). The synergistic modulation of these two enzymes decreases the levels of tetrahydrofolate and thymidylate in the parasite and therefore impairs the synthesis of nucleic acids and nuclear division.

SP is used in association with artesunate for the treatment of uncomplicated *P. falciparum* malaria. Artesunate is administered once a day for 3 days, while SP is given as a single dose on day 1. The major drawbacks of this ACT are the lack of availability as a fixed-dose combination, as well as the association with the emergence of drug resistance for prolonged SP treatment. DHFR is encoded by the *dhfr* gene located on chromosome 4, and several mutations that confer resistance to pyrimethamine have been identified, including Cys50Arg, Asn51Ile, Cys59Arg, Ser108Thr/Asn, and Ile164Leu. Particularly, the mutation at position 108 is known to be crucial for pyrimethamine resistance. In DHPS, five mutations that are responsible for sulfadoxine resistance have been identified: Ser436Ala/Phe, Ala437Gly, Lys540Glu, Ala581Gly, and Ala613Ser/Thr [24, 37].

At therapeutic doses, SP is well tolerated. Side effects are common to sulfonamide antibacterials, such as gastrointestinal disorders, headache, and skin reactions that might be severe or fatal in some cases [61].

### 2.3.6 Piperaquine

Piperaquine (Fig. 4) is a bisquinoline derivative that shares chemical features and mechanism of action with chloroquine. However, similar to other quinolines, piperaquine is effective against chloroquine-resistant strains most likely thanks to its bulky structure that is unsuitable for binding to efflux proteins that confer resistance to chloroquine.

Piperaquine is recommended for the therapy of uncomplicated malaria by different plasmodia in combination with dihydroartemisinin, although it might be also used for the treatment of severe malaria. The dihydroartemisinin/piperaquine ACT is available for oral administration as a fixed-dose combination with a ratio of 1:8, to be given once a day for 3 days in adults >25 kg.

Since piperaquine is known to prolong the QT interval, administration to patients with cardiac issues or congenital QT prolongation should be avoided [62]. Besides, the ACT is generally well tolerated with mild side effects such as nausea, diarrhea, and vomiting [63].

## 2.4 *Non-artemisinin-Based Drug Combinations*

The third edition of the WHO guidelines for malaria treatment suggests the use of ACT in the treatment of uncomplicated malaria, based on strong evidence of their efficacy in impairing parasite replication and prevention of drug resistance [49]. Nevertheless, the arsenal of drugs to treat malaria is composed of additional chemical entities and drug combinations devoid of the artemisinin scaffold, endowed with historical or bioactivity interest, which are described below.

### 2.4.1 Amodiaquine + Sulfadoxine-Pyrimethamine (AQ + SP)

Individual drugs and their use in ACT are described in Sects. 2.3.3 and 2.3.5, respectively. Their combination is used for the prevention of seasonal malaria in children <6 years in areas with remarkable seasonal transmission of the disease. The non-artemisinin-based combination is administered once a month for 4 months during the period of highest transmission of malaria, providing a decreased morbidity and mortality in treated children [64, 65]. In addition, the drug combinations proved to be highly effective in a randomized clinical trial in Uganda, thus becoming a low-cost potential alternative to chloroquine in Africa [50].

### 2.4.2 Atovaquone-Proguanil

This combination is very efficacious in the treatment of multidrug-resistant *P. falciparum* malaria, even in very resistant strains, having a cure rate of 98%. Atovaquone (Fig. 5) is a naphthoquinone derivative that acts as an inhibitor of plasmodial mitochondria electron transport at the cytochrome bc1 complex, depolarizes the mitochondrial membrane potential, inhibits dihydroorotate dehydrogenase enzyme that results in inhibition of the synthesis of nucleic acid and ATP [66]. The single mutation in the gene encoding cytochrome b (cytB) confers atovaquone resistance when it is used as a monotherapy. Proguanil hydrochloride (Fig. 5) acts as an inhibitor of DHFR. The combination of these two drugs stops parasitic deoxythymidilate synthesis [67, 68].

### 2.4.3 Quinine + Antibiotics (Doxycycline and Clindamycin)

Quinine is the preferred antimalarial drug for pregnant women and the treatment of multidrug-resistant malaria [37]. In combination with antibiotics, quinine has improved antimalarial efficacy and decreased side effects [69], and it is nowadays considered as a second-line option that is an alternative to ACTs when the latter are not available. The combination with the tetracycline derivative doxycycline (Fig. 5) is partially effective against the liver-stage of *Plasmodium* spp. and slows the

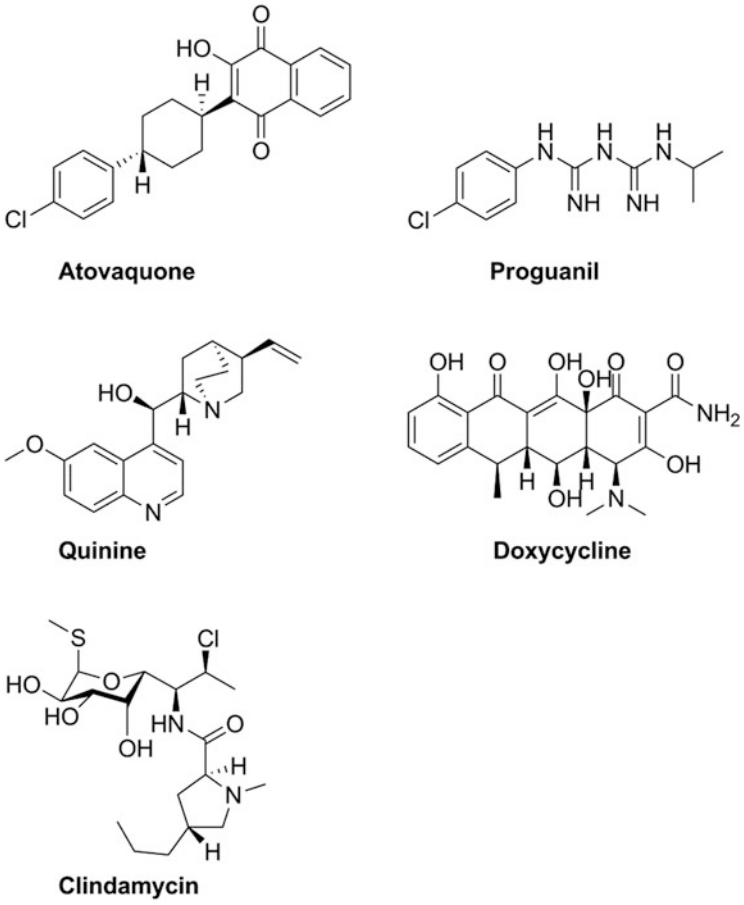


**Fig. 5** Chemical structure of antimalarial drugs used in non-artemisinin-based drug combinations

activity of blood schizontocidal agents. The use of this antibiotic has some advantages as easy absorption, enhanced solubility in lipids, and high stability [70, 71]. The main drawback of this combination strategy is the requirement of frequent administrations, i.e. every 8 h for a week, which decreases the overall patients' compliance and adherence to treatment protocols. Moreover, the use of this antibiotic is highly contraindicated in pregnant and breastfeeding women and children [72, 73]. Concerning the emergence of drug resistance, the mutations associated with *pfmdr1* in chloroquine have been found also in quinine resistance [74].

Clindamycin (Fig. 5) is another antibiotic with an average of 4–6 days of action clearance time. It can be used as monotherapy, but the dosage has to be twice a day for at least 5 days. It has several side effects such as nausea, vomiting, vaginal itching, heartburn, and stomachache. Its combination with quinine overcomes most of these safety issues, also providing a rapid action. The clindamycin-quinine

combination is suitable for the treatment of both children and pregnant women, with an administration protocol that is reduced to 7 days [75, 76]. However, in late pregnancy the clindamycin-quinine combination should be used with caution and only if alternatives are not available, in order to avoid the risk of hypoglycemia due to quinine.

## 3 Conclusion

Malaria is one of the most serious health problems worldwide, which affects mostly underdeveloped countries. Every year, approximately 400,000 deaths are due to malaria, mostly involving children <5 years old. Several campaigns have been launched by recognized international organizations for the prevention, control, and eradication of malaria. Unfortunately, most of them have failed so far (e.g., eradication campaigns, and the WHO 1998 campaign to have malaria over in 2010), with the number of total malaria cases reaching a plateau in the last 5 years. Also, the number of deaths per year is decreasing, with a slope that is significantly lower than expected. Particularly, African and Asian countries with a high burden of the disease recorded the worst outcomes in the fight against malaria. Several parameters contribute to these failures, such as logistic and organizational problems, shortage of funding, lack of effective and low-cost drugs, scarce adherence to treatment protocols, and the emergence of drug resistance to available therapeutic options. This latter is particularly penalizing, as the level of resistance to the former first-line drug chloroquine reached 90% in malaria-endemic countries, while resistance to other therapeutics such as the SP combination has reached up to 60%. At present, first-line choices for the therapy of malaria are represented by combinations between fast-acting artemisinin derivatives and longer-acting synthetic drugs, namely ACTs. These combinations are designed to improve the therapeutic efficacy and to decrease the susceptibility towards drug resistance. However, most of these drugs have been developed within the framework of a single research program dating back to the late 1970s (Fig. 6) [21].

In the last years, efforts have been developed to obtain new antimalarial chemotypes [77–79], some with dual-stage activity [80–82], as well as compounds with new mechanisms of action [83–85]. However, still a very limited number of potent antimalarial agents have been identified. One example is the spiroindolone NITD609, which has entered clinical trials in 2012 and has been the first drug candidate endowed with a different mechanism of action among antimalarial drugs that reached Phase IIa against malaria in the last 20 years [86]. This compound combines good pharmacokinetic and pharmacodynamics properties, without cytotoxicity [87]. Moreover, NITD609 proved to be highly efficient against the blood-stage of *P. falciparum* and *P. vivax* wild-type and multidrug-resistant strains, as well as in decreasing the oocyte count during the sexual stage in the mosquito [88, 89].

Currently, a number of vaccines and novel drug combinations are being developed [90–92]. In particular, over 20 vaccine constructs are currently being evaluated

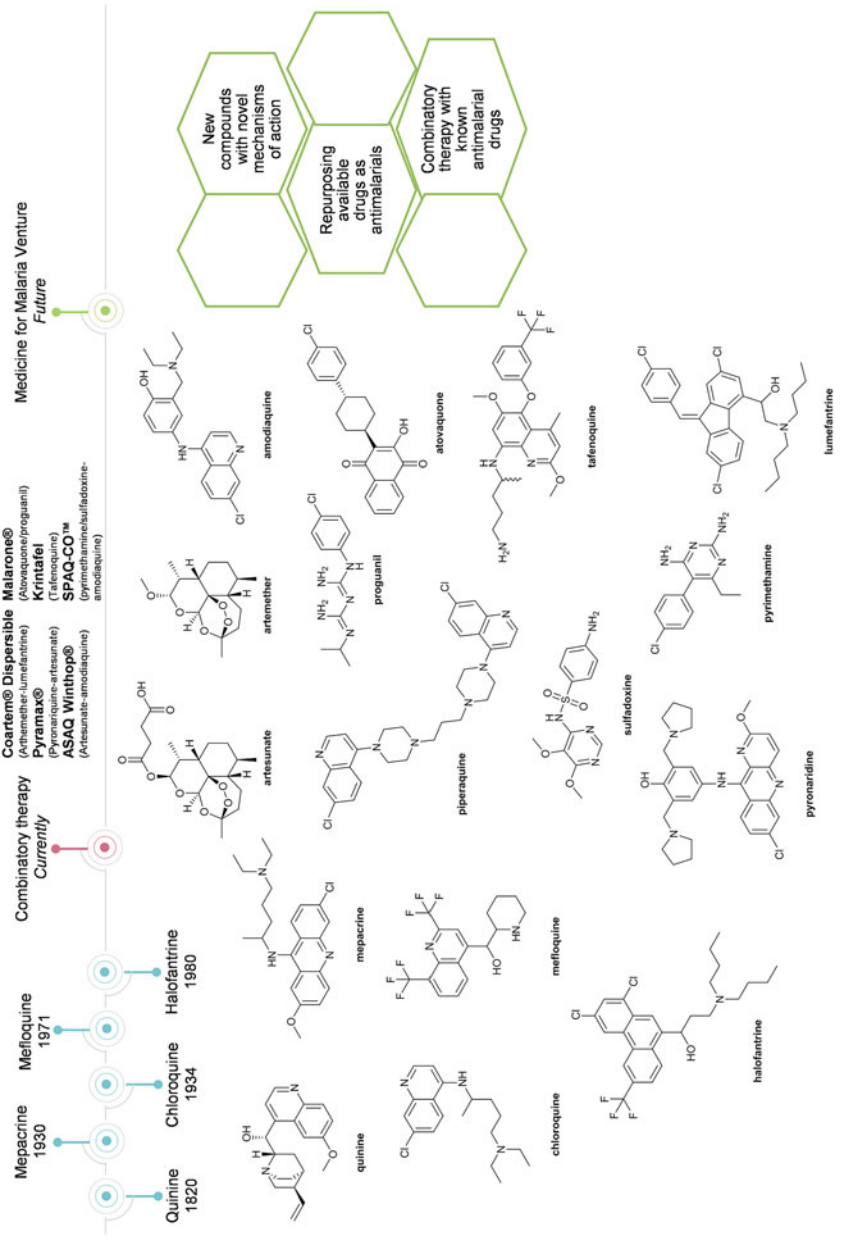


**Fig. 6** The past, present, and future of the treatment of malaria

in clinical trials or are in advanced preclinical development [93]. Among them, the world's first malaria vaccine named RTS,S/AS01 from GlaxoSmithKline has been approved by the European Medicines Agency (EMA) in 2015 with the trade name Mosquirix for the vaccination of young children, together with established antimalarial interventions [94, 95]. This vaccine acts against *P. falciparum*, the deadliest malaria parasite and the most prevalent in Africa. To date, the RTS,S vaccine is the first and the only option able to reduce malaria cases in young children by decreasing significantly the number of cases of uncomplicated and severe life-threatening malaria.

In addition to vaccines development, pharmaceutical companies are also contributing to the decrease of the cost of antimalarial drugs. Of note, given the efficacy of ACTs despite their higher costs compared to monotherapies or non-artemisinin drug combinations, Novartis decreased the price of Coartem by 50% since 2001. Nevertheless, these efforts are not enough as underlined by the current status of research and efforts against malaria. The WHO has recently launched the Global Technical Strategy for Malaria 2016–2030, which aims to reduce at least 90% of the incidence and mortality and to eliminate malaria in 35 countries by 2030 [96]. The achievement of these goals requires significant investments from multiple bodies including governments and health organizations [97].

In the current scenario, the emergence of resistance to first-line drugs represents a serious risk, and new effective and cheap drugs continue to be urgently needed [20]. However, still few efforts are devoted to antimalarial drug discovery compared to other global health threats. The COVID-19 pandemic represents a paradigmatic example. Indeed, after less than 1 year since the start of the pandemic, an antiviral drug is available (i.e., remdesivir), thanks to repurposing approaches, while a specific monoclonal antibody and additional drugs not interfering with viral replications have been repurposed for the management of COVID-19 patients [26]. Moreover, several vaccines have been approved for COVID-19 and are being administered at the global level, clearly evidencing that concerted and intersectorial efforts can efficiently respond to global health issues.

This critical analysis led to draw attention to the lack of concrete and focused actions against malaria. International organizations and national health systems should coordinate the efforts of academic and industrial researchers and provide the required financial resources to develop effective weapons against malaria rapidly, similar to what has been done for COVID-19.

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**Informed Consent**: All procedures in this chapter were not performed with human participants.

**Ethical Approval**: All procedures in this chapter were not performed neither with human participants, nor any other animals.

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# η-Class Carbonic Anhydrases as Antiplasmodial Drug Targets: Current State of the Art and Hurdles to Develop New Antimalarials


**Clemente Capasso and Claudiu T. Supuran**

## Contents



**Abstract** *Plasmodium falciparum* is responsible for the most severe and life-threatening form of malaria. The exceptionally high impact of malaria on human health is related to the ability of the parasites responsible for this disease to modify their genome to evade the human immune system and resist drug therapies. The lack of efficient treatments and acquired resistance to the existing therapies has stimulated efforts to identify new therapeutic targets to fight malaria. *P. falciparum,* during its

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exponential growth and replication in the erythrocytes, needs purines and pyrimidines for DNA/RNA synthesis, which are de novo synthesized from $HCO_3^-$, ATP, and glutamine. $HCO_3^-$ is involved in the Plasmodia pyrimidine pathway and is generated from $CO_2$ through the action of metalloenzymes known as carbonic anhydrases (CAs). We will review the current state of the art for inhibiting the CA (PfCAdom) from *Plasmodium falciparum* using the classical CA inhibitors, such as sulfonamides and their bioisosteres, organic anions, as well as phenol compounds. Some of these showed effective nanomolar inhibitory effect for PfCAdom and could be considered as leads for finding new drug candidates possessing a different mechanism of action from the clinically used drugs to which a considerable degree of drug resistance has been reported.

**Keywords** Anions, Carbonic anhydrase, Eta-CA, Inhibitors, Malaria, Phenolic compounds, *Plasmodium falciparum*, Sulfonamides

# 1 Introduction

## *1.1 Protozoan Infections*

Each year, hundreds of millions of people are infected with disease-causing protozoa, particularly in tropical and subtropical regions of the world, because humidity and high temperatures provide the necessary conditions for vectors and protozoans growth [1, 2]. Several of these diseases are neglected because of their incidence in countries with little purchasing power or their low visibility [3–7]. It has been estimated that approximately one million die each year due to protozoan infections, such as Leishmaniasis, Chagas disease, and especially malaria [6–9]. Leishmaniasis is an infection provoked by protozoans belonging to the genus Leishmania. Among the many species and subspecies of such protozoa, *Leishmania donovani chagasi* causes visceral leishmaniasis [10]. Leishmaniasis is transmitted by the bite of infected female phlebotomine sand flies [11]. American trypanosomiasis, or Chagas disease, is caused by the parasite *Trypanosoma cruzi*. The infection was described in 1909 by the Brazilian physician Carlos Chagas (1879–1934) [12]. About eight million people worldwide are estimated to be infected by *T. cruzi* [4–7, 9]. Furthermore, because of growing population migration, the disease has spread to other continents [13]. Chagas disease is transmitted to humans by the infected feces of blood-sucking triatomine bugs, a vector for the *T. cruzi* parasite; however, other transmission routes are known, such as consumption of contaminated food and drink, congenital, and blood transfusions [13]. Chagas disease chemotherapy is limited to nifurtimox and benznidazole; both drugs were developed more than 30 years ago [13]. They are predominantly active during the acute phase of the disease. However, they have serious adverse effects because of their high toxicity and low efficacy, especially in the chronic phase [13]. Malaria, a mosquito-borne

disease of humans and other animal species, is caused by parasitic protozoa species belonging to the genus Plasmodium. Six different Plasmodium species infect humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, and the zoonotic *Plasmodium knowlesi* [14, 15]. Globally malaria afflicts more than 200 million people and kills about 600,000 annually, mainly young children in sub-Saharan Africa, with most deaths caused by *P. falciparum* infection [16]. Malaria parasites follow a complex lifecycle that involves an intermediate host such as humans and the definitive host, the mosquito vector [16–24]. Following injection of sporozoite stage parasites from an infected female Anopheles mosquito into a human host, Plasmodium parasites move to the liver and invade hepatocytes where they replicate to form merozoites that are ultimately released into the blood circulation [16, 22]. Plasmodium merozoites can then invade erythrocytes and undergo cycles of asexual replication within these cells, resulting in the malaria's clinical symptoms [16, 22]. During this part of the lifecycle, sexual stage gametocytes can also form. When a feeding female Anopheles mosquito is taken up, they can undergo sexual reproduction in the mid-gut of the mosquito (Fig. 1a). This ultimately results in the completion of the life cycle through the formation of sporozoites that can then be transferred to another individual by the mosquito vector during a blood meal (Fig. 1a) [16, 25, 26]. While the first generation RTS,S/AS01 (RTS,S) malaria vaccine will be employed in some regions in the future, the World Health Organization (WHO) remains cautious and recommends that other malaria preventions and treatment strategies continue, including the development of new drugs [27, 28].

Here, we will review the current state of the art for inhibiting the carbonic anhydrases (CAs, EC 4.2.1.1) from *Plasmodium falciparum* with the goal to develop antiprotozoal agents possessing a different mechanism of action from the clinically used drugs to which a considerable degree of drug resistance has been reported.

## *1.2 Existing Antimalarial Strategies*

Six different Plasmodium species infect humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, and the zoonotic *Plasmodium knowlesi* [29, 30]. *P. falciparum* is responsible for the most severe and life-threatening form of malaria. The antimalarial drugs represent a keystone of malaria control [31, 32]. They follow two main strategies to control the disease: a) limit the development of gametocytes blocking transmission to mosquitoes; b) prevent malaria in endemic populations through chemoprophylaxis, intermittent preventive therapy, and mass drug dispensation [31, 32]. The effectiveness of the antimalarial medicines is influenced by drug resistance due to different factors, such as the mutations in the drug transporter of *Plasmodium falciparum*, the resistance to quinine (the oldest antimalarial drug), chloroquine, antifolates, artemisinin family drugs and malarone, a potent inhibitor of the electron transport [31–33]. Artemisinin derivatives can cure in 7 days the most severe malaria types [33]. However, there is a

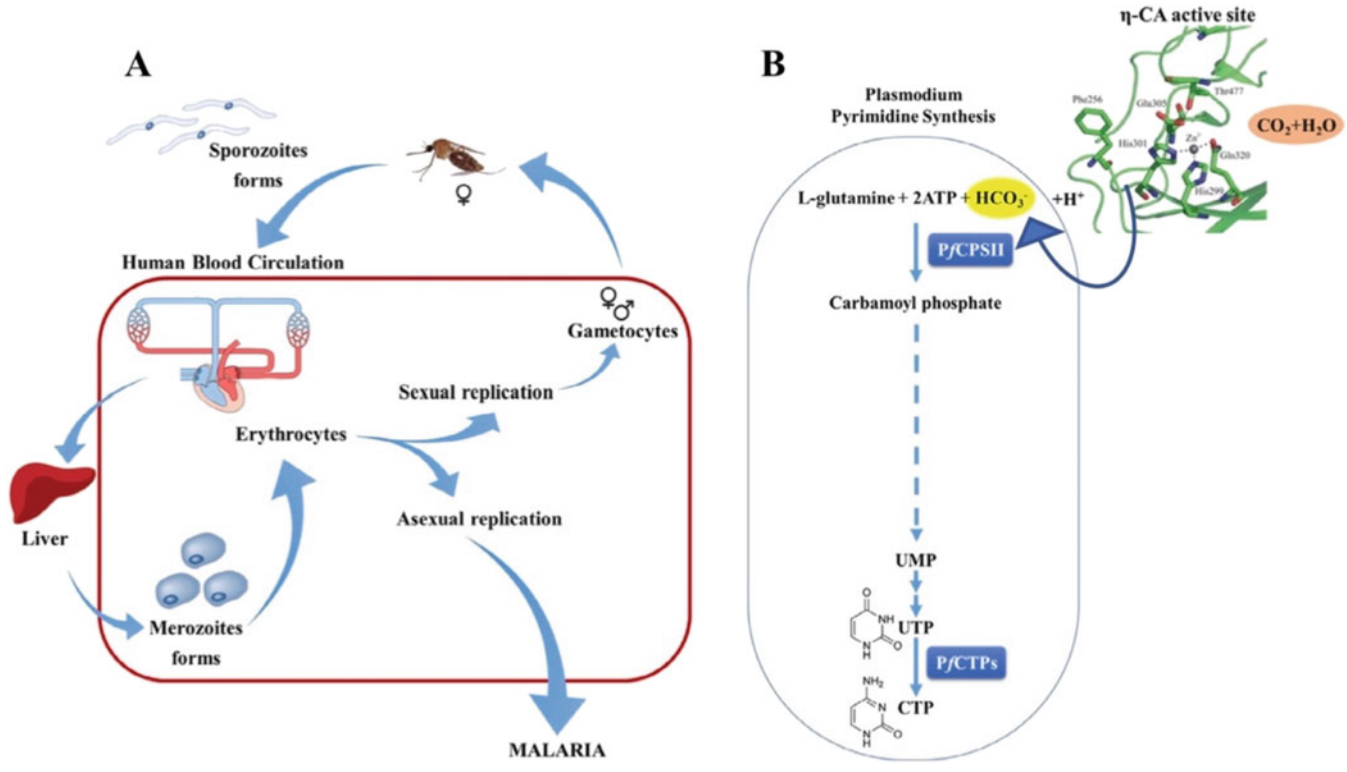


**Fig. 1** Panel (**a**), The life cycle of *Plasmodium falciparum*. Panel (**b**), Pyrimidine metabolism in *P. falciparum*. PfCPSII, carbamoyl phosphate synthetase II; PfCTPs, cytidine 50- triphosphate synthase. $HCO_3^-$, bicarbonate generated from $CO_2$ by the hydratase activity of the carbonic anhydrase

high risk that the disease will reappear [34] because parasites can continually develop resistance to each new class of drugs [33]. It has been seen that a combination of artemisinin derivatives can slow the development of resistance to other antimalarial drugs, but the combination of these drugs is quite expensive. In 2005, it was demonstrated that the administration of a variety of cheaper drugs, such as amodiaquine and sulfadoxine-pyrimethamine, can prevent recurrent malaria infections in the patients similarly when a combination of artemisinin derivatives is used [34]. Recently, it has been confirmed that one of the most effective strategies to contain malaria contagions is the use of mosquito nets treated with insecticides capable of killing Anopheles mosquitoes, which carry the plasmodium [35]. The researchers treated mosquito nets with an antimalarial already used in humans, the so-called atovaquone, which inhibits the normal functioning of mitochondria in *Plasmodium falciparum* cells, killing the parasite [35].

## 2 A New Druggable Enzyme from *Plasmodium falciparum*

The exceptionally high impact of malaria on human health is related to the ability of the parasites responsible for this disease to modify their genome to evade the human immune system and resist drug therapies [31–33]. The lack of efficient treatments and acquired resistance to the existing therapies has stimulated efforts to identify new therapeutic targets to fight malaria [17, 23, 36]. *P. falciparum,* during its exponential growth and replication in the erythrocytes, needs purines and pyrimidines for DNA/RNA synthesis (Fig. 1b) [17, 23, 36, 37]. Pyrimidines are present in only insignificant concentrations in human erythrocytes and *P. falciparum* does not have active pathways for the salvage of pyrimidines from the host. Thus, *P. falciparum* synthesizes pyrimidines de novo from $HCO_3^-$, adenosine-5-triphosphate (ATP), and glutamine (Gln). Intriguingly, $HCO_3^-$, which is the substrate of the carbamoyl phosphate synthetase II (*Pf*CPS II), the first enzyme involved in the Plasmodia pyrimidine pathway [37], is generated from $CO_2$ through the action of metalloenzymes known as carbonic anhydrases (CAs, EC 4.2.1.1) (Fig. 1b). Therefore, targeting plasmodium CAs for blocking the pyrimidine metabolic pathways might provide a promising route for novel drug development [17, 20, 22, 23, 38–41].

### *2.1 Carbonic Anhydrase*

CAs catalyze a common reaction in all life domains, the carbon dioxide hydration to bicarbonate and protons ($CO_2 + H_2O \Leftrightarrow HCO_3^- + H^+$) [42–45]. While writing this book chapter, eight CA-classes, indicated with the Greek letters α, β, γ, δ, ζ, η, θ, and ι, have been identified [43, 46, 47]. Although the eight different CA-classes originated from a common ancestor, they are phylogenetically distinct [43, 47]. The representative amino acid sequences of each CA-class show low sequence similarity,

different folds, and structures compared with the polypeptide chain of a CA belonging to the other classes [43, 47]. In contrast, the mechanism involved in the reversible hydration of the $CO_2$ is strictly conserved among all the CA-classes, demonstrating the pervasive convergent evolution of the CA superfamily [43, 47]. CAs are a group of metalloenzymes whose catalytic site contains a metal ion cofactor necessary for the enzyme catalysis [43, 44, 47, 48]. Usually, the $Zn^{2+}$ ion cofactor is coordinated by three amino acid residues from the protein. Simultaneously, the fourth ligand is a water molecule/hydroxide ion acting as the nucleophile in the catalytic enzyme cycle [43, 44, 48–51]. Some CA-classes can also coordinate metal ions different from $Zn^{2+}$, such as $Co^{2+}$, $Cd^{2+}$, $Fe^{2+}$, and $Mn^{2+}$. As described in the literature, α-, β-, δ-, η- and, perhaps θ-CAs use as ion cofactor the $Cd^{2+}$; γ-CAs the $Fe^{2+}$, although they can coordinate $Zn^{2+}$ or $Co^{2+}$, too [52–59]. The ζ-CAs are active with either $Cd^{2+}$ or $Zn^{2+}$incorporated into the same apoprotein and are defined as cambialistic enzymes [60–62]. More interesting is the discovery of a new CA-class, the ι-CA. It was identified for the first time in the genome of the marine diatom *Thalassiosira pseudonana*, and, surprisingly, the enzyme prefers as ion cofactor $Mn^{2+}$ [63]. The amino acid residues involved in the metal coordination differ between the CA-classes. For example, in the α-, γ-, δ- and, probably, θ-classes, the ion metal is coordinated by three His residues; in β- and ζ-CAs by one His and two Cys residues; by two His and one Gln residues in the η-class [36], and, probably, in the diatom ι-CAs the residues involved in the coordination of $Mn^{2+}$ are two His, one Asp and one Glu, although should be proved by biophysical technique [63]. From a structural point of view, as mentioned above, the representative belonging to one CA-class shows a different folding and structure compared with those of other CA-classes. α-CAs are usually active as monomers or dimers; β-CAs are active only as dimers, tetramers, or octamers. The γ-CAs must be trimers for accomplishing the catalytic function [53–55, 64]. γ-CA monomers are characterized by a tandemly repeated hexapeptide, which is crucial for the left-hand fold of the trimeric *β*-helix structures [65]. The X-ray structure of the θ-CAs resulted in very similar to the β-CAs [66]. The crystal structure of ζ-CA showed three slightly different active sites on the same polypeptide chain [60]. No information is available on the structures of δ-, η-, and ι-CAs. Intriguing, α-, η-, θ- and ι-CAs were reported to catalyze the esters/thioesters' hydrolysis, while no esterase activity was detected for the other CA families [51, 63, 67]. Intriguing is the distribution pattern of the CA-classes in the living organisms. CAs present in mammals belong to α-class [68, 69], plants and algae have α-, β-, γ-, δ-, and θ-classes; fungi encode for α- and β-CAs; protozoa for α-, β-, and/or η-CAs [47]. In metazoans, the α-CAs are the predominant enzymes showing $CO_2$ hydratase activity [70, 71]. In 2019, Gontero and coworkers reported that the genome of some bacteria contains genes with relevant homology to the diatom ι-class CA [63], and these new bacterial sequences were annotated in the data bank as oxidoreductases [63]. In 2020, Capasso and coworkers demonstrated that the bacterial ι-CA (acronym BteCAι) identified in the genome of *Burkholderia territorii* resulted in an excellent catalyst for the hydration of $CO_2$ to bicarbonate and protons [46]. Thus, α-, β-, γ-, and ι-CAs are the typical classes present in Bacteria [8, 21, 42–44, 46, 48, 72].

## 2.2 Plasmodium falciparum *CA*

Generally, pathogenic protozoa, such as *Plasmodium spp.*, *Trypanosoma cruzi,* and *Leishmania spp.* encode for α- β- or a new class of CAs, the η-CAs [19, 73–75]. The causative agent of human malaria, *Plasmodium falciparum*, was one of the first protozoa to be investigated for the presence of CAs [73]. The open reading frame of the malarial CA enzyme (*P. falciparum* CA, accession number AAN35994.2, PlasmoDB: PF3D7_1140000) encodes a 600 amino acid polypeptide chain, which showed important amino acid substitutions that differentiated the sequence of Plasmodium enzyme from those of other CA-classes [17]. In 2004, Krungkrai et al. [73] cloned a truncated form of this gene encoding for a polypeptide chain named PfCA1. It was formed by the 235 amino acid residues (amino acid residues from 221 to 445) with a theoretical molecular mass of 27.9 kDa. In 2015, De Simone et al. using the homology modeling demonstrated that the Krungkrai enzyme (PfCA1) did not include the amino acid residues from 182 to 220 and from 446 to 538, which could be modeled with known tridimensional CA structure [36]. Thus, this prompted us to consider a wider portion of the plasmodium η-CA (358 amino acid residues), which was named PfCAdom and had a molecular mass of 42.3 kDa. Again, the phylogenetic tree published in the paper by Del Prete et al. in 2014 evidenced that Plasmodia CAs clustered in a branch different from that of the α-CAs, although close to it, while they were well separated from the other CA-classes [17]. Based on these data, it has been hypothesized that Plasmodia CAs were the result of modifications of an ancestral δ-CA gene, which originated a new CA-class, which was denominated with the Greek letter η [17]. The three-dimensional model realized by De Simone et al. [36] shows that the metal ion coordination pattern of the η-CA from malaria producing protozoa *P. falciparum* is unique among all six genetic families encoding for such enzymes, comprising two His and one Gln residues, in addition to the water molecule/hydroxide ion acting as a nucleophile in the catalytic cycle (Fig. 2). Although the η- and α-CAs share many similar features, strongly suggesting the first ones to be evolutionary derived from the last, there are significant differences between the two families to allow some optimism for the drug design of selective inhibitors for the parasite over the host enzymes. However, these studies are still in their initial phase. Further work by X-ray crystallography should validate the model proposed to detect inhibitors with high affinity and selectivity for the η-CAs over the α-CAs [36].

## 2.3 *PfCA1 and PfCAdom Kinetic Parameters*

The recombinant polypeptide chain PfCAdom was prepared by designing a synthetic gene and heterologously expressed in *Escherichia coli* as a HisTag fusion protein [76]. Using the stopped-flow technique, the kinetic parameters were determined for the recombinant PfCAdom using $CO_2$ as a substrate. The activity of PfCAdom was

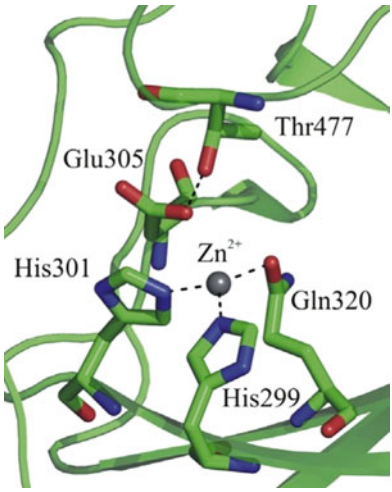


**Fig. 2** The active site of the PfCAdom resulted from homology modeling. The figure indicates the residues (His299, His301, Gln320) coordinated to the zinc ion, the residues Glu305 and Thr477, forming the Glu-Thr dyad. PfCAdom numbering system was used

compared to that of PfCA1 (Kungrai truncated form) and with other $\alpha$-CAs, such as the *Homo sapiens* isoforms hCA I and hCA II. The protozoan full-length domain (PfCAdom) showed a $k_{cat}$ of $3.8 \times 10^5$ $s^{-1}$ and a $k_{cat}/K_M = 7.2 \times 10^7$ $M^{-1}$ $s^{-1}$. Intriguing, the PFCAdom $k_{cat}/K_M$ resulted in one order of magnitude higher respect to that of the truncated form, PfCA1 ($k_{cat}$ of $1.4 \times 10^5$ $s^{-1}$ and a $k_{cat}/K_M = 5.4 \times 10^6$ $M^{-1}$ $s^{-1}$). This was expected since the truncated form lacked a Thr residue (Thr199 in hCA II corresponding to Thr 477 in PfCAdom), which is presumed to be crucial for catalysis and orienting $CO_2$ in the proper mode for the nucleophilic attack from the zinc-coordinated hydroxide. Interesting, both the truncated (235 aa) and the full-length (358 aa) enzymes resulted active on the SDS-Page when subjected to the protonography analysis [76].

## 3 *PfCA*dom Inhibition

### *3.1 The Most Investigated CA Inhibitors*

Together with the inorganic anions, sulfonamides are the most studied carbonic anhydrase inhibitors (CAIs) [51, 62, 77, 78]. Domagk discovered antimicrobial sulfonamides in 1935 [79], and they were the first antimicrobial drugs to be widely used in clinical settings. The first sulfonamide showing effective antibacterial activity was Prontosil, a sulfanilamide prodrug, the last compound being isosteric/isostructural with p-aminobenzoic acid (PABA), the substrate of dihydropteroate synthase [80]. In the following years, a range of analogs constituting the so-called sulfa drug class of antibacterials entered into clinical use [23, 76, 81–94]. **AAZ**, **MZA**, **EZA**, and **DCP** are systemically acting antiglaucoma CAIs. **DZA** and **BRZ** are antiglaucoma agents that function topically; **BZA** is an orphan drug of this

pharmacological class. **ZNS**, **SLT**, and the sulfamic acid ester **TPM** are widely used antiepileptic drugs. **SLP** and **IND** also belong to this class of pharmacological agents, together with the COX2 selective inhibitors **CLX** and **VLX**. **SAC** and the diuretic **HCT** are also known to act as CAIs. **FAM** is a competitive histamine $H_2$-receptor antagonist [95] and **EPA is** an inhibitor of the heme-containing enzyme, indoleamine 2,3-dioxygenase-1 (IDO1) [96]. Most of the sulfonamides, such as the clinically used derivatives **AAZ**, **MZA**, **EZA**, **DCP**, **DZA**, and **BZA**, bind in a tetrahedral geometry to the Zn(II) ion in the deprotonated state, with the nitrogen atom of the sulfonamide moiety coordinated to Zn(II) and an extended network of hydrogen bonds, involving amino acid residues of the enzyme, also participating in the anchoring of the inhibitor molecule to the metal ion [51, 62, 67]. The aromatic/heterocyclic part of the inhibitor interacts with the hydrophilic and hydrophobic residues of the catalytic cavity [51, 62].

Anions, such as inorganic metal-complexing anions or more complicated species such as carboxylates, are also known to bind to CAs [62, 67]. These anions may bind either the tetrahedral geometry of the metal ion or as trigonal–bipyramidal adducts [97]. Anion inhibitors are usually less effective than sulfonamides, which generally show $K_{Is}$ in a nanomolar range. Their investigation is essential for understanding the inhibition/catalytic mechanisms of these enzymes fundamental for many physiologic processes and designing novel types of inhibitors that may have clinical applications for managing a variety of disorders in which CAs are involved [62, 67].

## *3.2 Sulfonamide Inhibition Profile*

The PfCAdom protein was subject to an extensive inhibition study with sulfonamides and sulfamates for the detection of low nanomolar inhibitors, comparing them with the data of the truncated form PfCA1 and the human isoforms hCA I and II [76]. The sulfonamides CA inhibitors generally showed much weaker inhibitory activity against PfCAdom compared to PfCA1. The amino acid residues of the full-length amino acid sequence (PfCAdom), some of which present in the active site, are crucial for the functional architecture of the catalytic pocket. The best sulfonamide inhibitors for PfCAdom were acetazolamide, methazolamide, metanilamide, and sulfanilamide, with $K_{Is}$ in the range of 366–808 nM [76].

Famotidine (**FAM**), an antiulcer drug belonging to the $H_2$ antagonist class of pharmacological agents, was recently shown to potently inhibit human (h) and bacterial CAs [98]. It has been investigated the inhibitory effects of **FAM** against the protozoan enzyme from *Plasmodium falciparum*. The drug resulted in very efficacy with a $K_I$ of 142 nM [98], making it a possible lead or a potential agent for more detailed, in vivo investigations.

### 3.3 Anion Inhibition Profile

PfCAdom was generally less inhibited by most anions and small molecules compared to PfCA1 [86]. The best PfCAdom inhibitors were sulfamide, sulfamic acid, phenylboronic acid, and phenylarsonic acid, which showed $K_{Is}$ in the range of 9–68 $\mu$M, followed by bicarbonate, hydrogen sulfide, stannate, and *N,N*-diethyldithiocarbamate, which were submillimolar inhibitors, with $K_{Is}$ in the range of 0.53–0.97 mM [86].

### 3.4 *In Vitro Inhibition of* Plasmodium falciparum *Growth*

In 1998, Sein and Aikawa showed that the addition of CA inhibitors (CAIs) to a culture of *P. falciparum* provoked a remarkable reduction in parasitemia [99]. Successive reports illustrated that specific CA inhibition in *P. falciparum* and rodent parasite *P. berghei* produced the death of the parasite in vitro cultures [100]. Recently, from a high throughput screening of a GlaxoSmithKline (GSK) it has been demonstrated that primary sulfonamide (PS) chemotype, which is not currently used for malaria prevention or treatment, has antimalarial potential [101]. The GSK screen results led to the compilation of the Tres Cantos Antimalarial Set (TCAMS) [101]. Thirty-one of these compounds were investigated for their ability to selectively inhibit the in vitro growth of *Plasmodium falciparum* asexual stage malaria parasites. Fourteen of these compounds were found to have submicromolar activity (IC50 = 0.16–0.89 mM) and a modest selectivity index (SI) for the parasite versus human cells (SI > 12 to >43) [101]. These compounds were assessed for the inhibition of recombinant PfCAdom. Unfortunately, the PfCAdom inhibition activity did not correlate with antiplasmodial potency, suggesting that the asexual intraerythrocytic stage antiplasmodial activity of the PS compounds is likely unrelated to PfCAdom inhibition [101].

## 4 PfCAdom Inhibition with Phenolic Compounds

### 4.1 Phenolic Compounds

As above reported, the sulfonamides and their bioisosteres are the most potent class of CAIs, but these chemotypes commonly show a weak isoform selectivity for the subset of human CAs and enzymes belonging to distinct CA-classes. This prompts the research of other chemotypes, which can be selective modulators for targeting the CA from pathogens. For example, a series of compounds, such as N-nitrosulfonamides, phenols, and natural polyphenols, resulted in excellent inhibitors of the $\beta$-CAs from pathogens over the human $\alpha$-CAs. In this context, a series of

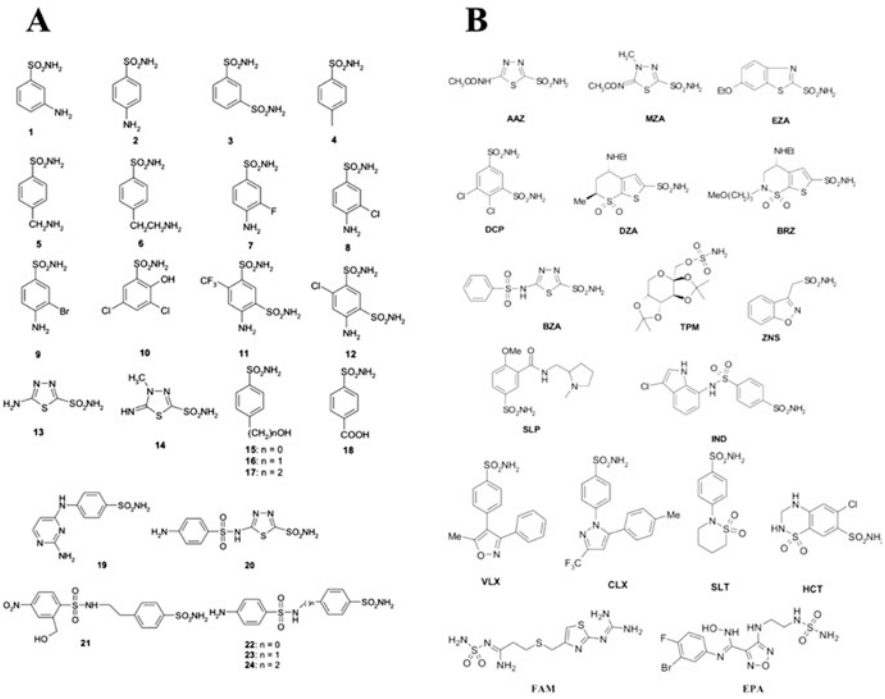


**Fig. 3** Sulfonamides tested as CAIs against PfCA: (**a**) Simple derivatives. (**b**) Sulfonamides, sulfamates and sulfamides in clinical use or in clinical development for the treatment of other CA-mediated diseases

phenolic derivatives (**1–22**, Fig. 3) was assessed for the inhibition of PfCAdom in search of novel leads for drug candidates and selective modulation over human isozymes [102]. Some derivatives showed effective submicromolar inhibition of PfCAdom ($K_{Is}$ 0.62–78.7 $\mu$M). Moreover, a subset of compounds demonstrated a significant selectivity for targeting PfCAdom over the human CAs [102]. These results are significant for identifying new potent and selective inhibitors of PfCAdom, which could be considered as leads for finding drug candidates in the treatment of malaria.

## 5 Conclusions

The full spread of the pathogenic resistance to the standard drugs represents a leading threat to human health. A primary strategy to combat it consists of identifying novel therapeutic targets and anti-infectives with alternative mechanisms of action. The inhibition of CAs from pathogens was shown to produce impairment of the microorganism growth and virulence. Significant interest is being dedicated to

the *Plasmodium falciparum* CA because PfCAdom is engaged in the production of $HCO_3^-$, which is a precursor of the pyrimidine biosynthetic pathway of the plasmodium. PfCAdom inhibition could represent an efficient strategy for developing new pharmacological agents against malaria. For this reason, inhibitors, such as sulfonamides and their bioisosteres, organic anions, and phenol compounds, were used to assess in vitro the inhibition of PfCAdom. Some of these showed effective nanomolar inhibition versus PfCAdom, which could be considered as leads for finding new drug candidates for more detailed in vivo investigations as well as in the treatment of malaria.

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# Management of *Entamoeba histolytica* Infection: Treatment Strategies and Possible New Drug Targets


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## Contents



**Abstract** *Entamoeba histolytica* infection, amoebiasis, is a major cause of morbidity and mortality in developing countries. It is also a significant causative agent of traveler's diarrhea. It has been estimated that amoebiasis may affect 10% of the global population. The most common infection route is via ingestion of contaminated food and water. About 90% of infected individuals are asymptomatic, but the infection may also lead to severe complications, such as colitis with bloody diarrhea, liver abscesses, and colonic perforation. The classical gold standard for diagnosis is the detection of trophozoites from stool samples by microscopy, although this method is labor-intensive and has low sensitivity. Several other diagnostic methods, based on parasite culture, serologic tests, antigen detection, and polymerase chain reaction, have been developed. In the future, multiplex PCR methods will be widely used for the simultaneous detection of various pathogenic microorganisms including *E. histolytica*. Treatment of amoebic colitis typically involves a combination therapy with the so-called luminal agents (paromomycin, diloxanide furoate, iodoquinol) combined with tissue amoebicides (metronidazole, tinidazole). Even though the present treatment options are mostly effective, new drugs are needed to treat all patients with amoebiasis, and different vaccine candidates are under development to eradicate *E. histolytica* from population.

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## 1 Biology and Pathogenesis of *Entamoeba histolytica*

*Entamoeba histolytica* is a unicellular pathogenic protozoan causing amoebiasis which mainly occurs as an intestinal infection [1, 2]. Bloody diarrhea (amoebic colitis) and liver abscess are the most common consequences [3]. The clinical manifestations are often divided into three groups depending on the symptoms and spreading of the parasite in the human body: (1) Intraluminal amoebiasis covers the first weeks of the infection when there are no symptoms, but the diagnosis could be made. (2) Amoebic colitis is the most common appearance of the disease which includes diarrhea, sometimes bloody stools, fever, abdominal cramps, and weight loss. (3) The most severe form is a disseminated amoebiasis in which the parasite forms abscesses in internal organs, although the intestinal symptoms may be absent or mild [4–6]. The most common site for abscess is the liver, and other extraintestinal lesions have been reported in the brain, lung, and peritoneum [7, 8].

*E. histolytica* is closely related to another species of the *Entamoeba* family, *E. dispar*. For years it was considered possible to be an asymptomatic carrier of *E. histolytica* [6, 9]. Detailed studies showed that asymptomatic carriers were, in fact, infected with *E. dispar* instead of *E. histolytica*. Thus, it is now concluded that *E. histolytica* infection leads to a symptomatic disease, but nevertheless, the symptoms may be mild [10].

The life cycle of *E. histolytica* has two different stages including cysts and trophozoites. Transmission occurs via fecal-oral route. Infection is usually contracted by eating food contaminated with quadrinucleated cysts, more rarely directly by person-to-person contact [6]. Excystation occurs in the small intestine where one cyst releases eight motile trophozoites. Trophozoites migrate to the large intestine, adhere the mucous wall through multi-unit Gal/GalNAc lectins, form new cysts, and invade through the intestine wall [11]. *E. histolytica* is capable of lysing human tissues, killing immune effector cells by contact-dependent cytolysis and with amoebapores and can degrade the host extracellular matrix with cysteine proteases. Trophozoites are easily destroyed if they encounter the gastric fluid or environment outside the human body. However, cysts may survive up to weeks outside the body with an ability to cause infection. Hence, the cysts secreted to stool are ready to transmit amoebiasis to other people [6].

## 2 Epidemiology of *E. histolytica* Infection

Worldwide, *E. histolytica* infections lead to the death of over 55,000 people annually [12, 13] and approximately 50 million people have a symptomatic infection each year [4, 14]. According to the World Health Organization *E. histolytica* is the third leading cause of death from parasitic disease; only malaria and *Schistosoma mansoni* cause more mortality [15]. Fortunately, there is some indication that the mortality rates of amoebiasis are gradually decreasing.

Amoebiasis is endemic in tropical and subtropical areas, which mostly involve developing countries. However, globalization and traveling brings the parasite to developed countries, and the prevalence has been estimated to be as high as 4% in the USA [4]. For comparison, the seroprevalence is up to 42% in rural areas of Mexico. Higher incidence and prevalence figures are strongly associated with the lower quality and availability of sanitation in the area.

## 3 Diagnosis of *E. histolytica* Infection

There are multiple methods with different characteristics to diagnose amoebiasis. The classical golden standard has been microscopy. Although it is labor-intensive and requires skilled technicians, its simplicity and low cost have outweighed the obvious limitations. Therefore, microscopy still remains widely used, especially in resource-limited laboratories of endemic, high-prevalence areas [16]. Microscopy has low sensitivity and specificity, and it is time-consuming as it often requires multiple samples to reach the final diagnosis [17].

A wide variety of quantitative real-time polymerase chain reaction (qPCR) assays have been recently developed for the diagnosis of enteric viral, bacterial, and parasitic agents. PCR has also become a widely recommended method as the primary tool for diagnosing *E. histolytica* infection. To reach a more comprehensive view of the infection from a single specimen, there has been a trend towards multiplex approach that allows simultaneous identification of multiple pathogens [18, 19]. Several multiplex gastrointestinal pathogen panel tests are already commercially available, some of them involving fully integrated robotic systems incorporating DNA extraction, amplification, detection, and analysis directly from stool samples [20, 21]. Food and Drug Administration (FDA, USA) has approved several gastrointestinal panels involving *E. histolytica* detection to clinical practice and recommends them as golden standard, and the World Health Organization (WHO) also advocates PCR as the primary method [4, 22]. On the one hand, PCR is sensitive (sensitivity 92–100%), specific (specificity 89–100%), and rapid, but on the other hand, it requires equipment, kits, and an educated technician [20, 23].

Stool antigen detection, serology, culture, isoenzyme analysis, and point-of care (POC) tests are other options which have been widely investigated [4, 17, 20]. Often none of them alone leads to the final diagnosis, but they are certainly useful as

complementary tests. As an example, stool antigen detection has been used as a complementary test for microscopy, which can overcome the limited sensitivity and specificity of the classical microscopy test. Serology is particularly useful for detecting the cases with extraintestinal infections, when the stool sample was negative. Unfortunately, serology does not separate an active infection from past infection [4]. Culture and isoenzyme analyses are additional tools to differentiate *E. histolytica* from *E. dispar*, but the success rate of the culture is only 50–70%, the risk of false negative is high, and the methods are time-consuming. Therefore, PCR has largely replaced culture in diagnostic use [17]. POC tests are typically commercial test assays which are based on antigen detection, serology, or PCR. Their characteristics and costs vary enormously. Nonetheless, a sensitive and specific POC may bring significant advantage in endemic areas, allowing mass screening of population.

## 4 Current Treatment Options for *E. histolytica* Infections

The medication used to treat amoebiasis can be divided in three groups depending on their point of action: intraluminal, tissue, and mixed amoebicides [24]. Intraluminal amoebicides are effective against cysts in the gut, tissue amoebicides treat the symptomatic disease in intestines and other tissues, and mixed treatments have both actions.

Recommended treatment options according to Haque et al. are described in Table 1 [25]. The traditional treatment against *E. histolytica* infection is metronidazole, a widely used antiparasitic and anti-anaerobic bacteria drug [26]. The recommended first-line treatment includes three daily doses of 750 mg of metronidazole for 5 (or 7)–10 days or three daily doses of 800 mg of tinidazole for 5 days [25, 27]. Oral administration is usually sufficient even in invasive infections as the bioavailability of metronidazole is approximately 80%. Nevertheless, intravenous administration is also an option in hospital setting, if the response to oral treatment was found inadequate.

Metronidazole is effective against trophozoites but is usually inadequate to eradicate cysts from the gut [26]. Therefore, in the management of all forms of invasive disease, including amoebic colitis, the standard recommendation is to give a tissue amoebicide (metronidazole or tinidazole) followed by an intraluminal amoebicide (diloxanide furoate, paromomycin, or iodoquinol) [26, 28]. This treatment procedure would optimally eradicate both the live parasites and intraluminal cysts. It is notable, however, that some controversy still exists whether cyst eradication is always needed after metronidazole or tinidazole treatment, especially in endemic areas, where re-infection is frequent [29]. The increased complexity of combination regimens, additional drug costs, more frequent side events, and the restricted availability of intraluminal amoebicides on the local market, all reduce compliance with combination therapy.

**Table 1** Suggested treatment options for amoebiasis according to Haque and coworkers [25]

| Diagnosis and drug | Adult dosage | Pediatric dosage |
|---|---|---|
| *Amoebic liver abscess* | | |
| Metronidazole | 750 mg orally × 3, 7–10 days | 35–50 mg/kg/day in 3 divided doses, 7–10 days |
| | *Or* | |
| Tinidazole | 800 mg orally × 3, 5 days | 60 mg/kg/day (maximum 2 g), 5 days |
| | *Followed by a luminal agent* | |
| Paromomycin | 25–35 mg/kg/day in 3 divided doses, 7 days | 25–35 mg/kg/day in 3 divided doses, 7 days |
| | *Or second-line agent* | |
| Diloxanide furoate | 500 mg orally × 3, 10 days | 20 mg/kg/day in 3 divided doses, 10 days |
| *Amoebic colitis* | | |
| Metronidazole | 750 mg orally × 3, 7–10 days | 35–50 mg/kg/day in 3 divided doses, 7–10 days |
| | *Followed by a luminal agent as for amoebic liver abscess* | |
| *Asymptomatic intestinal colonization* | | |
| Paromomycin | 25–35 mg/kg/day in 3 divided doses, 7 days | 25–35 mg/kg/day in 3 divided doses, 7 days |
| | *Or second-line agent* | |
| Diloxanide furoate | 500 mg orally × 3, 10 days | 20 mg/kg/day in 3 divided doses, 10 days |

As all pharmaceutical agents, metronidazole has adverse side effects, for instance nausea, diarrhea, loss of appetite, and metallic taste in the mouth [27, 30]. Comparison of metronidazole and tinidazole has not revealed any major difference concerning the subjective side-effect profiles of these drugs [31]. Notably, metronidazole inhibits the action of hepatic CYP2C9 enzyme which leads to many undesirable interactions with other drugs, such as frequently used anticoagulant warfarin [32, 33]. Hence, the inhibition of CYP2C9 may lead to decreased or increased concentrations of other drugs in the blood stream, potentially leading to drug-related adverse side effects or loss of action.

It is noteworthy that only few creditable clinical trials exist in the medical literature considering the pharmacological treatment of *E. histolytica* infection. Gonzales and coworkers published a meta-analysis of randomized controlled trials of antiamoebic drugs given alone or in combination, compared with placebo or another antiamoebic drug, for amoebic colitis [29]. In total, they were able to include 41 trials (4,999 participants) which met the inclusion criteria. However, many trials were old and only one used adequate randomization and allocation concealment, was blinded, and analyzed all randomized participants. Moreover, the diagnostic methods used in those trials were not always reliable. Despite these uncertainties, they concluded that compared with metronidazole, (1) tinidazole may be more

effective in reducing clinical failure, (2) tinidazole may be associated with fewer adverse events, and (3) combination drug therapy may be more effective for reducing parasitological failure.

*E. histolytica* resistance against metronidazole has been considered rare. Wassmann et al. [34] and Samarawickrema et al. [35] induced resistance in axenic *E. histolytica* cultures up till lethal doses of metronidazole. The mechanism of resistance has been shown to involve increased activity of iron-containing superoxide dismutase (Fe-SOD) and peroxiredoxin and decreased expression of flavin reductase and ferredoxin 1 [34, 35]. The activation of Fe-SOD is usually a reaction to various stress inducing situations, for instance overpopulation of cells, and thus not only the drug effect of metronidazole [35]. In the case of metronidazole, the activation of SOD may be linked to the protection of microorganisms from a variety of toxic radicals.

## 5 Future Therapeutics and Vaccine Development

Sulfolipid metabolism is necessary for the parasitic lifestyle of *E. histolytica* [36]. The sulfate activation is performed through two sequential reactions producing adenosine 5′-phosphosulfate (APS) and 3′-phosphoadenosine 5′-phosphosulfate (PAPS) with the catalysts ATP sulfurylase (AS) and APS kinase (APSK), respectively. PAPS is used as a sulfate donor in a variety of reactions which provide crucial molecules for trophozoite proliferation and encystation. Sulfate activation takes place in mitochondrial-related organelles called mitosomes from where PAPS is transferred to cytosol where sulfolipids are generated with the catalyzing help of sulfotransferases (SULTS) and sulfatases (SF). From these enzymes the APSK has been considered the most promising target of antiamoebic drug development, as it is unique to *E. histolytica* physiology in the early steps of sulfate activation. 2-(3-fluorophenoxy)-N-[4-(2-pyridyl)thiazol-2-yl]-acetamide (A-D-11), 3-phenyl-N-[4-(2-pyridyl)thiazol-2-yl]-imidazole-4-carboxamide (A-H-11), and auranofin have been found to halt trophozoite proliferation as well as encystation [37, 38]. A-D-11 and A-H-11 have no cytotoxic effect in human cells, in contrast to auranofin which is, in fact, already in human use as an oral drug for rheumatoid arthritis [37, 39]. Auranofin also inhibits thioredoxin reductase, enhancing sensitivity of trophozoites to reactive oxygen-mediated killing [38]. Thioredoxin reductase of *E. histolytica* (EhTrxR) is an important enzyme in the redox system and for intracellular oxygen detoxification. Martínez-Pérez and coworkers recently showed that rabeprazole, a proton pump inhibitor, inhibits the EhTrxR enzyme [40]. Rabeprazole also affected amoebic proliferation and several other functions required for parasite virulence. In a hamster model of liver infection, sublethal rabeprazole concentration (600 μM) promoted parasite death. The authors concluded that the molecular structure of rabeprazole can be useful as a scaffold to design new amoebicides.

Nitazoxanide is a novel antiparasitic agent, which has been shown to be effective against *E. histolytica* in both the intraluminal and invasive forms of infection and has been suggested to represent a potential successor to metronidazole [41].

Flavonoids, such as kaempferol, catechin, and isoquercitrin, have antiamoebic activity, which has been demonstrated only in vitro [42]. Therapeutic dosage, administration route as well as pharmacokinetics and dynamics are yet to be determined.

*E. histolytica* has a single β-carbonic anhydrase (EhiCA) [43]. EhiCA was produced as a recombinant protein which was used in kinetic and inhibition studies using different sulfonamides and anions [44, 45]. Bua et al. discovered 4-hydroxymethyl/ethyl-benzenesulfonamide to have the best inhibitory action against EhiCA ($K_I$s of 36–89 nM) with weaker inhibition impact on human carbonic anhydrase I and II ($K_I$s of 21 μM and 125 nM, respectively) [44]. Several carbonic anhydrase inhibitors, clinically used for other conditions, were also tested. Among these compounds, acetazolamide, methazolamide, ethoxzolamide, and dichlorphenamide showed good inhibitory effects ($K_I$s of 509–845 nM), while they also inhibited efficiently human CA I and II ($K_I$s ranging 8–1,200 nM) [44]. Thus, these compounds provided no selectivity against EhiCA. In addition, some anions had good inhibition properties: sulfamide, phenylarsonic acid, phenylboronic acid, and fluorosulfonate showed $K_I$s of 28 μM, 38 μM, 47 μM, and 86 μM, respectively [45]. Furthermore, their inhibitory effects against human carbonic anhydrase I and II were weaker than against EhiCA ($K_I$s ranging 310 μM to 49.2 mM), which makes them slightly selective against the amoeba carbonic anhydrase. These results clearly opened new avenues for further investigations to determine the effects of carbonic anhydrase inhibitors in vivo and to design novel compounds specifically targeting β-carbonic anhydrases.

As *E. histolytica* is an important cause of morbidity and mortality especially in low-income countries, the need of vaccine is real. Humans and non-human primates are the only reservoirs of *E. histolytica,* which makes the eventual goal to eradicate the disease plausible [10]. *E. histolytica* triggers many immune pathways of the host, which has further led to attempts to develop a vaccine against this parasite [2, 24, 46]. A Gal/GalNAc lectin-based vaccine has been the most widely investigated candidate; also a serine-rich *E. histolytica* protein and an attenuated strain of *E. histolytica* have been investigated in rodent models. Nevertheless, none of these theoretically promising vaccines have reached clinical trials. We hope that the interest in novel vaccines against *E. histolytica* will increase along with the new era in vaccinology that has recently been witnessed during the COVID-19 pandemic. The eradication of *E. histolytica* should be considered both an important goal for better global health and an investment for the global sustainable development goals.

## 6 Concluding Remarks

*Entamoeba histolytica* is the third leading cause of mortality of parasite infections, which causes pressure to have tools for rapid diagnosis as well as affordable and effective treatment. The clinical manifestation of amoebiasis varies from an asymptomatic infection to colitis and even to life-threatening invasive infection. Fortunately, we have good treatment options for different clinical situations, although there is already some indication of emerging drug resistance. Vaccination would represent the most effective option to reduce the global disease burden in long term, but no such preventive option is available at this moment.

**Compliance with Ethical Standards** *Conflict of Interest:* The authors declare that they have no conflict of interest.

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# *Trichomonas vaginalis* Pharmacological Treatment


**Seppo Parkkila**

## Contents



**Abstract** Trichomoniasis is the most common sexually transmitted protozoan infection, which has been treated for several decades using nitroimidazoles, mainly metronidazole and tinidazole. Both drugs are still recommended and resistance to them has fortunately been a relatively rare phenomenon. Resistant or tolerant cases exist, however, side effects are also notable. Therefore, novel compounds with different mechanism of action are urgently needed. It is encouraging that several novel and innovative leads have been introduced. They will hopefully help us to develop novel antitrichomonal agents to fight harder against this parasitic disease in the future.

**Keywords** Diagnosis, Drug, Therapy, Treatment, Trichomonas vaginalis, Trichomoniasis

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## 1 Prevalence and Symptoms of Trichomoniasis

According to the World Health Organization, *Trichomonas vaginalis infection,* trichomoniasis, is considered the most common sexually transmitted, curable protozoan infection worldwide (https://www.who.int/bulletin/volumes/85/4/06-031922/en/). According to one large study with 4,057 participants from the USA, the prevalence of trichomoniasis was 0.5 and 1.8% among males and females, respectively [1]. In another report, *T. vaginalis* had infected over 11% of women aged ≥40 years, and the infection prevalence was found to be associated with the age of patients, their place of residence, ethnicity, socioeconomic status, and number of sex partners [2, 3]. The high prevalence in the general population has mostly been reported in the U.S. cohorts. Lower prevalence estimates were found in Britain. From urinary samples of 4,386 individuals *T. vaginalis* infection was detected in only seven women and no men, giving a weighted prevalence estimate of only 0.3% [4]. As mentioned above, there may be several confounding factors which could explain the lower infection prevalence reported in that study.

Trichomonas is a motile, protozoan organism with a size comparable to leukocytes [5] (Fig. 1). It has at least four flagella that drive cell locomotion. The infection leads to increased vaginal pH and release of cytotoxic proteins that destroy the epithelial lining.

Diagnosis and treatment of trichomoniasis are challenging since the majority of *T. vaginalis* infections in women are asymptomatic [6], and as untreated, the infection may last for months or years. Trichomoniasis is associated with several

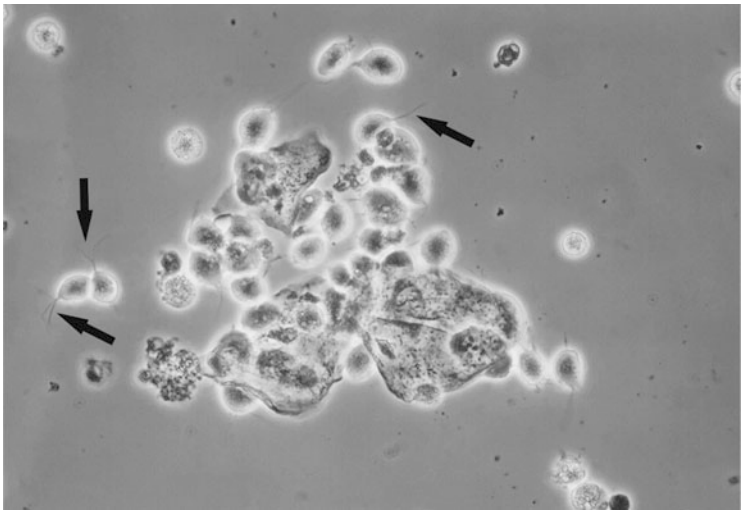

**Fig. 1** Wet-mounted vaginal discharge specimen showing several *T. vaginalis* parasites, indicative of trichomoniasis. Some flagella are visible in the parasites (arrows). Courtesy of CDC/Joe Miller (https://phil.cdc.gov/Details.aspx?pid=14500)

adverse consequences, such as preterm birth, delivery of a low-birth weight infant, and infection with a *human immunodeficiency virus* (HIV) [3].

The common symptoms of *T. vaginalis*-infected women include a copious, yellow-green, frothy, and malodorous vaginal discharge, vulvar irritation, pruritus, dysuria, dyspareunia, and post-coital bleeding [7, 8]. Speculum examination may reveal a "strawberry cervix" sign due to punctate hemorrhages of the ectocervix. In addition, erythematous and edematous vaginal walls due to vaginitis may be observed. In men, the infection may present as urethritis, epididymitis, or prostatitis [8]. Trichomoniasis is readily passed between sex partners. In a study of 540 women with trichomoniasis and 261 of their male partners, 71.7% of partners got the infection and 77.3% of them were asymptomatic [9]. An additional challenge is that trichomoniasis sometimes exists with other sexually transmitted diseases, such as HIV, *Chlamydia trachomatis*, and *Neisseria gonorrhoeae* infections [2]. However, the rates of *T. vaginalis*, *C. trachomatis*, and *N. gonorrhoeae* coinfection were low (<1.3%) when studied in the whole population. In a Kenyan cohort, trichomoniasis showed a 1.52-fold increased risk of HIV-1 acquisition [10]. In another large cohort from Uganda and Zimbabwe, statistical analysis indicated an odds ratio 2.74 for HIV in *T. vaginalis*-positive cases [11]. Based on several studies, it can be concluded that *T. vaginalis* infection increases both the transmission and acquisition of HIV among women, and that successful treatment for trichomoniasis can reduce the transmission of HIV [12].

## 2 Diagnosis of Trichomoniasis

The clinical features of trichomoniasis are variable and thus not sufficiently sensitive or specific to allow trichomoniasis diagnosis based upon signs or symptoms alone. The laboratory diagnostics are based on several alternative laboratory tests, including the detection of motile trichomonads on the wet preparation of a vaginal swab (wet mount), *T. vaginalis* culture, polymerase chain reaction (PCR) test, transcription-mediated amplification test, and rapid antigen test [13, 14]. Pap smear is not recommended as a diagnostic method for trichomoniasis due to its low sensitivity and specificity [7]. The wet mount microscopy is the low cost, classical method which has also shown low sensitivity in the range of 40–60% [5]. In one study, sensitivities of 50.8%, 75.4%, 82.0%, and 98.4% were reported for wet mount microscopy, culture, rapid antigen test, and transcription-mediated amplification test, respectively [15]. Other studies have further confirmed that rapid antigen testing outperforms both *T vaginalis* culture and wet mount as a diagnostic tool [16, 17]. Recently, PCR detection has become the gold standard for diagnosis [18] and can be used with different specimens including both urine and vaginal samples [19]. Tayoun and coworkers introduced a multiplex PCR assay for the simultaneous testing of *T. vaginalis*, *N. gonorrhoeae*, and *C. trachomatis*, which are the three most common sexually transmitted diseases worldwide [19]. They demonstrated that the multiplex assay is rapid, sensitive, and highly suitable for clinical

laboratories. Point-of-care tests have been developed to facilitate rapid, accurate, and affordable diagnostics especially in emergency departments [20]. In the future, self-testing might become a potential option. Interestingly, >99% of 209 young women aged 14–22 years correctly performed and interpreted their own self-test result using the OSOM Trichomonas Rapid Test (Sekisui Diagnostics, Framingham, MA), with a high correlation with clinicians´ interpretations [21]. Recently, Xiu and coworkers developed a sophisticated 23-plex PCR coupled with matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) assay that can simultaneously detect 11 different agents, including the eight clinically relevant pathogens related to sexually transmitted infections (*T. vaginalis,* HSV-1, HSV-2, *N. gonorrhoeae, C. trachomatis, Treponema pallidum, Mycoplasma genitalium*, and *Haemophilus ducreyi*) and three controversial microorganisms as pathogens (*Mycoplasma hominis, Ureaplasma urealyticum*, and *Ureaplasma parvum*) [22]. They concluded that, based on its high sensitivity and specificity, the method could serve as a high-throughput screening tool for detecting mixed, sexually transmitted infections.

## 3 Pharmacological Treatment of Trichomoniasis

Patients with trichomoniasis need prompt and effective treatment as soon as the diagnosis has been confirmed. Metronidazole and other nitroimidazoles, including tinidazole, ornidazole, nimorazole, and carnidazole, have been used as effective drugs [23]. Despite their widespread use for decades, resistance has been relatively rare. The treatment guidelines of Centers for Disease Control and Prevention (CDC) clearly state that nitroimidazoles are currently the only class of antimicrobial medications known to be effective against *T. vaginalis* infections (https://www.cdc.gov/std/tg2015/trichomoniasis.htm) [24].

Three different regimens for standard treatment have been presented: (1) a single 2 g dose of metronidazole, (2) a single 2 g dose of tinidazole, and (3) 500 mg metronidazole twice a day for 7 days. Benefits of tinidazole include a longer half-life, it reaches higher levels in serum and the genitourinary tract, and it has shown slightly fewer gastrointestinal side effects compared with metronidazole [25, 26]. A meta-analysis of 54 randomized or quasi-randomized controlled trials indicated that almost any nitroimidazole drug given as a single dose or over a longer period results in parasitological cure in at least 90% of cases [23]. The oral single dose treatment with a higher dose is associated with more frequent side effects than the longer treatment with a lower dose. Because of the limitations of studies, it was not possible to rank tinidazole superior to metronidazole or vice versa. Tinidazole tends to have a longer half-life in the body, and thus it may possess longer duration effect when compared with metronidazole. If metronidazole failed, tinidazole should be the other drug to be used [5].

As special cases, patients with known HIV infection should receive 500 mg metronidazole twice daily for 7 days [5]. Treatment seems to be justified also in

pregnant women diagnosed with trichomoniasis [5, 27, 28]. If left untreated, the infection can result in adverse outcomes; especially, the rate of preterm delivery is increased. The preferred drug is metronidazole and women should stop breastfeeding during treatment [5].

## 4 Nitroimidazole Resistance of *T. vaginalis*

Nitroimidazole resistance has emerged as a real threat that may challenge the well-established treatment regimens for trichomoniasis in the future. Graves and coworkers recently conducted a systematic review of the literature on the mechanisms of 5-nitroimidazole resistance [29]. Based on the data from 58 articles, drug resistance is higher to metronidazole (2.2–9.6%) than tinidazole (0–2%).

Graves and colleagues [29] pointed out that the mechanisms for drug resistance may have already existed in 1962, when Robinson described the first case of metronidazole-resistant trichomoniasis [30]. Interestingly, the resistance mechanisms of nitroimidazoles in T. vaginalis are probably different than in some bacteria. In *Trichomonas,* the resistance to 5-nitroimidazoles appears to be more relative than absolute. Graves et al. [29] further pointed out that the failure of clinical treatment may be more of a function of drug tolerance rather than developed drug resistance. One clinical observation supporting this concept is that *T. vaginalis* infections, unresponsive to the currently recommended doses of metronidazole, can often be treated by increasing dosages [31].

## 5 Future Perspectives

Even though both metronidazole and tinidazole are well-documented and successfully used drugs against *T. vaginalis*, the resistance of the parasite to metronidazole has emerged as a notable issue [29, 32]. Side effects are another concern in some patients. Therefore, novel treatment options are highly desired. Recently, Lee and coworkers reviewed several compounds showing some promising results against *T. vaginalis* [33]. The compounds among many others, showing micromolar or even nanomolar $IC_{50}$ values, included such as nitrothiazole and benzothiazole derivatives [34], hybrid conjugates with incorporated β-lactam, triazole and isatin nuclei [35, 36], and thiosemicarbazone-derived ruthenium metal complexes [37]. Recently, Supuran's, De Simone's, and Parkkila's groups introduced a novel enzyme, *T. vaginalis* β-carbonic anhydrase (TvaCA1), which can be targeted using several known carbonic anhydrase inhibitors [38, 39]. These studies are reviewed in another chapter of this book.

**Compliance with Ethical Standards** *Conflict of Interest:* The author declares that he has no conflict of interest.

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*Ethical Approval:* This chapter does not contain any studies with human participants or animals performed by the author.

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# Beta-Carbonic Anhydrase 1 from Trichomonas Vaginalis as New Antiprotozoan Drug Target


**Claudiu T. Supuran, Anna Di Fiore, Seppo Parkkila, and Giuseppina De Simone**

## Contents



**Abstract** *Trichomonas vaginalis* is a unicellular parasite responsible for trichomoniasis, which is one of the world's leading sexually transmitted infections (STIs). The diagnosis and effective treatment of trichomoniasis has become an extremely important goal for global health, due to the increasing experimental evidences showing the relationship between trichomoniasis and other critical pathologies and the appearance of resistance to the existing pharmacological treatments. Consequently, in recent years research of novel drug targets for fighting this STI has seen an increased interest. In this scenario and considering recent experimental evidences which indicate Carbonic Anhydrases (CAs) as potential targets for the treatment of protozoan parasitic diseases, our group focused the attention on TvaCA1, a β-CA from *T. vaginalis*, carrying out a complete biochemical, structural, and kinetic characterization of this enzyme. In this chapter, we will summarize these

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studies, showing that this enzyme is a druggable target and that its selective inhibition is feasible with the aim to obtain new anti-trichomoniasis drugs.

**Keywords** Drug design, Drug target, Inhibition, *Trichomonas vaginalis*, β-carbonic anhydrase

## 1 Introduction

Globally, more than one million sexually transmitted infections (STIs) occur each day which are associated with significant morbidity and mortality. In this scenario, a very important issue is the identification of a strategy to manage STI epidemic potential, changing patterns of the diseases, preventability of the diseases, and their social and economic effects. Critical points for the prevention and control of STIs include rapid diagnosis and early therapeutic treatment of infections in order to interrupt the transmission and reduce the untreated cases [1].

The human-infective parasite *Trichomonas vaginalis* is the causative agent of the most widespread non-viral STI worldwide, namely the trichomoniasis [2]. Based on the "Report on global sexually transmitted infection surveillance," there were roughly 156 million new infections every year attributable to *T. vaginalis* pathogen [3, 4]. In particular, *T. vaginalis* is a flagellate protozoan that affects lower female genital tract, with a prevalence of 2.1% in reproductive age-women [5], and the prostate epithelium [6, 7]. Clinically, symptoms of *T. vaginalis* infection can appear weeks, months, or years after the initial infection [8, 9] and include mild to moderate inflammation of the cervix, vagina, and urethra [10–12]. However, since many cases are asymptomatic, millions of *T. vaginalis* infections remain undiagnosed and therefore untreated [3], suggesting that asymptomatic individuals represent an infection risk to their sexual partners.

The recent increased interest for the treatment of trichomoniasis infection depends on the observation that this STI can cause serious damage in some physiological or pathological conditions. In fact, it has been observed that during pregnancy trichomoniasis could be responsible for premature rupture of the amniotic sac, preterm labor, and delivery of a low birth weight [13, 14], while infected individuals could show increased susceptibility to human immunodeficiency virus (HIV) acquisition and/or transmission [15]. In addition, previous investigations suggested that *T. vaginalis* infection might determine an increased risk of cervical neoplasia [16]. Recently, the effect of this pathogen as risk factor for persistence and/or progression of low-grade cervical precancerous lesions has also been evaluated in HIV-1 positive women, showing that *T. vaginalis* infection can negatively modulate this pathological condition [17]. On the other side, infections in men that occur mainly in colonization of the prostate can increase the risk of aggressive prostate cancer [18]. In particular, the pathogen expresses a protein involved in

cellular pathways linked to inflammation and cell proliferation, thus contributing to the initiation and progression of cancer.

In this scenario, enabling an early diagnosis and an effective pharmacological treatment of *T. vaginalis* infection represent very important goals for global health protection. At present, trichomoniasis management involves the use of only one type of drug, the 5-nitroimidazoles, which could be associated with several side effects [19–21]. However, the major disadvantage of this therapy is the rapid appearance of resistance and a natural drug tolerance among a certain population of *T. vaginalis* isolates [22]. Since no alternative treatments were so far developed, a large number of research studies have been focused on the identification of new and more effective compounds acting as anti-infective drugs. In particular, different classes of molecules have been tested in vitro for their antiparasitic action, as an example 5-nitroimidazole derivatives, benzo[f]cinnoline N-oxide and metronidazole containing dual active chemical group, while other potential therapeutic agents have been identified by screening natural compounds [2].

An alternative therapeutic strategy to counteract STIs caused by non-viral microorganisms involves the identification of new molecular targets. The cloning of the genomes of many pathogenic microorganisms offered the possibility of exploring alternative pathways for inhibiting virulence factors or proteins essential for their life cycle.

Carbonic Anhydrases (CAs, EC 4.2.1.1), ubiquitous metallo-enzymes which catalyze the reversible hydration of carbon dioxide to bicarbonate and proton [23], have been recently proposed as new potential targets for the treatment of protozoan parasitic diseases. Indeed, convincing data in the literature strongly indicate that the inhibition of CA activity in various parasites leads to a damage of parasite growth and virulence, causing a significant anti-infective effect [24]. Eight evolutionarily unrelated CA families have been so far identified (α-, β-, γ-, δ-, ζ-, η-, θ-, and ι-CAs), which do not show significant structural homology with each other [23, 25–27]; therefore, the possibility to develop specific inhibitors of one family, which do not interact with the other ones, is highly feasible [28]. Interestingly, only α-CAs are present in humans, whereas many parasites contain β-, γ-, and η-CAs; thus, suggesting that the latter enzymes could represent excellent target molecules for the development of drugs free of potential side effects [24]. For this reason in recent years many studies describing the production, characterization, and inhibition of parasite β-, γ- and/or η-CAs have been carried out [29–48].

In this context we recently reported the expression in *E. coli* of a new β-CA from *T. vaginalis* (TvaCA1) and its structural, biochemical, and kinetic characterization [36, 38, 48]. In this chapter, we will summarize these studies, showing that this enzyme is a druggable target and that its selective inhibition is feasible with the aim to obtain new anti-trichomoniasis drugs.

## 2 TvaCA1 Biochemical, Kinetic, and Structural Characterization

TvaCA1 was expressed in *E. coli* and purified with high yield, in order to carry out a complete biochemical, kinetic, and structural characterization [38]. In the same paper size exclusion chromatography and light scattering experiments were described showing a dimeric quaternary structure for the recombinant protein. These data were in agreement with the observation that β-CAs always show a dimeric structure that in some cases can arrange in higher oligomers such as tetramers, hexamers, or octamers. The $CO_2$ hydration activity was also measured by means of a stopped-flow instrument revealing a rather high catalytic efficiency comparable to that of human (h) CA I, protozoan β-CAs from *Entamoeba histolytica* [49] and *Leishmania donovani chagasi* [39] and some prokaryotic β-CAs, such as *Salmonella enterica* [50] and *Legionella pneumophila* [51] (Table 1), but lower than that of the highly efficient isoform hCA II.

Subsequently, the crystallographic structure of the enzyme was determined [38], showing the typical α/β-fold previously observed for other βCAs [53–65], consisting of a central five-stranded β-sheet core, formed by four parallel (with strand order 2-1-3-4) and one antiparallel β-strands (β5) and flanking helices (Fig. 1a). Two monomers associated to form the biologically relevant dimer, originating an extended β-sheet core of ten β-strands and generating a total buried surface area at the dimeric interface of 4,366 $Å^2$ (Figs. 1b and 2a) [38].

In this dimer, the N-terminal helix of each monomer extends away from the rest of the molecule, making extensive contacts with the other monomer. The dimeric structure contained two active sites, which were located in clefts at the dimeric interface, each one containing a zinc ion on the bottom coordinated by three protein residues, namely Cys37, His96, and Cys99, and a solvent molecule/hydroxide ion (Fig. 2). Interestingly, these active sites were scarcely accessible when compared to those of hCAs, which are situated in a large and deep conical cavity (Fig. 3).

**Table 1** Kinetic parameters of TvaCA1. For comparison, kinetic parameters of hCA I, hCA II, and other representative protozoan and bacterial β-CAs are reported

| Enzyme | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_M$ ($M^{-1}$ $s^{-1}$) | $K_I$ (**AAZ**) (nM) |
|---|---|---|---|
| TvaCA1 [38] | $4.9 \times 10^5$ | $8.0 \times 10^7$ | 391 |
| hCA I [52] | $2.0 \times 10^5$ | $5.0 \times 10^7$ | 250 |
| hCA II [52] | $1.4 \times 10^6$ | $1.5 \times 10^8$ | 12 |
| EhiCA [49] | $6.7 \times 10^5$ | $8.9 \times 10^7$ | 509 |
| LdcCA [39] | $9.4 \times 10^5$ | $5.9 \times 10^7$ | 92 |
| SenCA1 [50] | $1.0 \times 10^6$ | $8.3 \times 10^6$ | 59 |
| SenCA2 [50] | $7.9 \times 10^5$ | $5.2 \times 10^7$ | 84 |
| LpnCA1 [51] | $3.4 \times 10^5$ | $4.7 \times 10^7$ | 76 |
| LpnCA2 [51] | $8.3 \times 10^5$ | $8.5 \times 10^7$ | 72 |

**AAZ** = acetazolamide, EhiCA = *Entamoeba histolytica* β-CA, LdcCA = *Leishmania donovani chagasi* β-CA, SenCA = *Salmonella enterica* β-CA, LpnCA = *Legionella pneumophila* β-CA

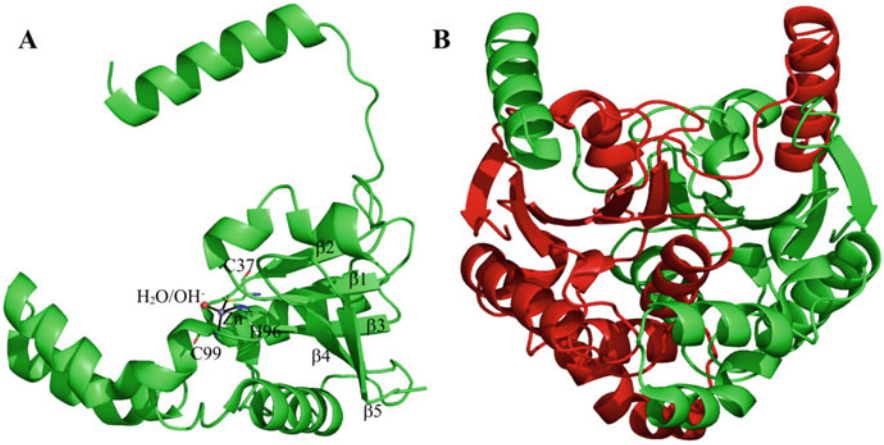


**Fig. 1** Ribbon representation of the TvaCA1 monomer (**a**) and dimer (**b**) with one monomer colored in red and the other in green [38]

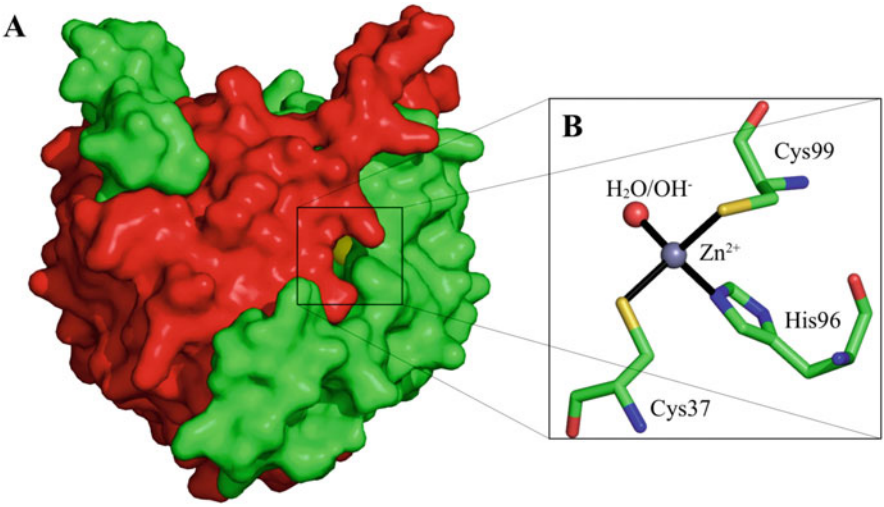


**Fig. 2** (**a**) Surface of TvaCA1 dimeric structure, with the two monomers shown in green and red. (**b**) Enlarged view of the active site of the enzyme, with the zinc coordinated by two Cys, one His and one water molecule/hydroxide ion [38]

This is an important difference that can be exploited for the design of inhibitors selective for the protozoan enzyme with respect to the human CAs, which represent an off-target for the development of antiparasitic drugs.

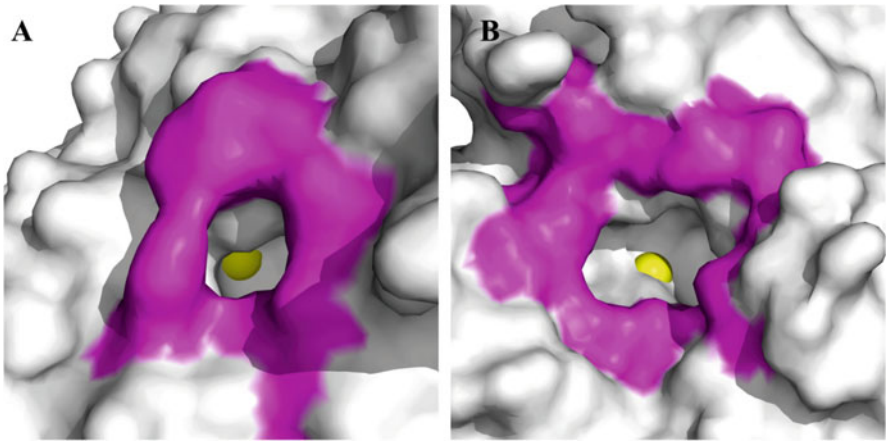


**Fig. 3** Surface representation of (**a**) TvaCA1 and (**b**) hCA II (chosen as a representative human isoform) showing the active site accessibility of these two enzymes [38]. Residues delimiting the rim of the active site cavity are colored in magenta, while the catalytic zinc ions are depicted as yellow spheres

## 3 TvaCA1 Inhibition with Anions and Sulfonamides

In order to gain information on the molecules which could be used for the development of TvaCA1 selective inhibitors, a wide range of inorganic anions and small molecule compounds were investigated for their inhibition properties against the parasitic enzyme and the results were compared to those obtained for hCA I and hCA II with the same set of molecules (Table 2) [36]. These studies identified thiocyanate, cyanide, selenate, selenocyanate, divanadate, and N,N-diethyldithiocarbamate as sub-millimolar inhibitors, and sulfamide, sulfate, phenylboronic acid, and phenylarsonic acid as micromolar inhibitors. The latter two compounds were the most interesting ones, since they were rather selective for TvaCA1 with respect to hCA I and hCA II (see Table 2), thus emerging as lead compounds for the development of new antiprotozoan drugs with a different mechanism of action [36].

Subsequently a series of simple aromatic/heterocyclic primary sulfonamides as well as several clinically approved/investigational such drugs for a range of pathologies were also investigated (Fig. 4, Table 3) [48] and compared with the results previously obtained for the off-target hCA II. Interestingly out of the 40 tested derivatives only 14 were able to inhibit TvaCA1, the remaining 26 being ineffective up to 50 μM concentration in the assay system. Among the inactive compounds were the clinically used agents **DCP**, **DZA**, **BRZ**, **BZA**, **TPM**, **ZNS**, **SLP**, **IND**, **VLX**, **CLX**, **SLT**, **SAC**, and **HCT**. Most of them possessed rather bulky scaffolds, thus explaining why they could not enter the scarcely accessible TvaCA1 active site and on the contrary were very good inhibitors of hCA II, possessing a very large and well accessible active site. Similar considerations can be done for the compounds of the series **1–24**; among these, molecules with very bulky scaffold were completely

**Table 2** Inhibition constants of anion and small molecule inhibitors against hCA I, hCA II and the protozoan enzyme TvaCA1 measured by $CO_2$ hydrase stopped-flow assay

| Inhibitor | $K_I$ (mM) | | |
|---|---|---|---|
| | hCA I[a] | hCA II[a] | TvaCA1[b] |
| $F^-$ | >300 | >300 | >100 |
| $Cl^-$ | 6 | 200 | 8.7 |
| $Br^-$ | 4 | 63 | 7.7 |
| $I^-$ | 0.3 | 26 | 2.1 |
| $CNO^-$ | 0.0007 | 0.03 | 2.2 |
| $SCN^-$ | 0.2 | 1.60 | 0.71 |
| $CN^-$ | 0.0005 | 0.02 | 0.91 |
| $N_3^-$ | 0.0012 | 1.51 | 3.3 |
| $HCO_3^-$ | 12 | 85 | 7.1 |
| $CO_3^{2-}$ | 15 | 73 | >100 |
| $NO_3^-$ | 7 | 35 | 3.7 |
| $NO_2^-$ | 8.4 | 63 | 1.8 |
| $HS^-$ | 0.0006 | 0.04 | >100 |
| $HSO_3^-$ | 18 | 89 | >100 |
| $SnO_3^{2-}$ | 0.57 | 0.83 | 3.9 |
| $SeO_4^{2-}$ | 118 | 112 | 0.39 |
| $TeO_4^{2-}$ | 0.66 | 0.92 | 8.5 |
| $OsO_5^{2-}$ | 0.92 | 0.95 | >100 |
| $P_2O_7^{4-}$ | 25.77 | 48.50 | >100 |
| $V_2O_7^{4-}$ | 0.54 | 0.57 | 0.64 |
| $B_4O_7^{2-}$ | 0.64 | 0.95 | >100 |
| $ReO_4^-$ | 0.110 | 0.75 | >100 |
| $RuO_4^-$ | 0.101 | 0.69 | 1.2 |
| $S_2O_8^{2-}$ | 0.107 | 0.084 | >100 |
| $SeCN^-$ | 0.085 | 0.086 | 0.64 |
| $CS_3^{2-}$ | 0.0087 | 0.0088 | >100 |
| $Et_2NCS_2^-$ | 0.00079 | 0.0031 | 0.49 |
| $SO_4^{2-}$ | 63 | >200 | 2.8 |
| $ClO_4^-$ | >200 | >200 | >100 |
| $BF_4^-$ | >200 | >200 | >100 |
| $FSO_3^-$ | 0.79 | 0.46 | >100 |
| $NH(SO_3)_2^{2-}$ | 0.31 | 0.76 | 2.2 |
| $H_2NSO_2NH_2$ | 0.31 | 1.13 | 0.044 |
| $H_2NSO_3H$ | 0.021 | 0.39 | 0.083 |
| Ph-$B(OH)_2$ | 58.6 | 23.1 | 0.093 |
| Ph-$AsO_3H_2$ | 31.7 | 49.2 | 0.062 |

[a]From reference De Simone and Supuran [66]
[b]From reference Urbanski et al. [36]

ineffective as CA inhibitors, whereas those incorporating more compact, simple benzenesulfonamide/thiadiazole sulfonamide scaffolds with few compact substituents (such as **1–4**, **7, 13–15** and **MZA**) were able to better accommodate within the enzyme active site producing micromolar inhibition. However, also in this case the

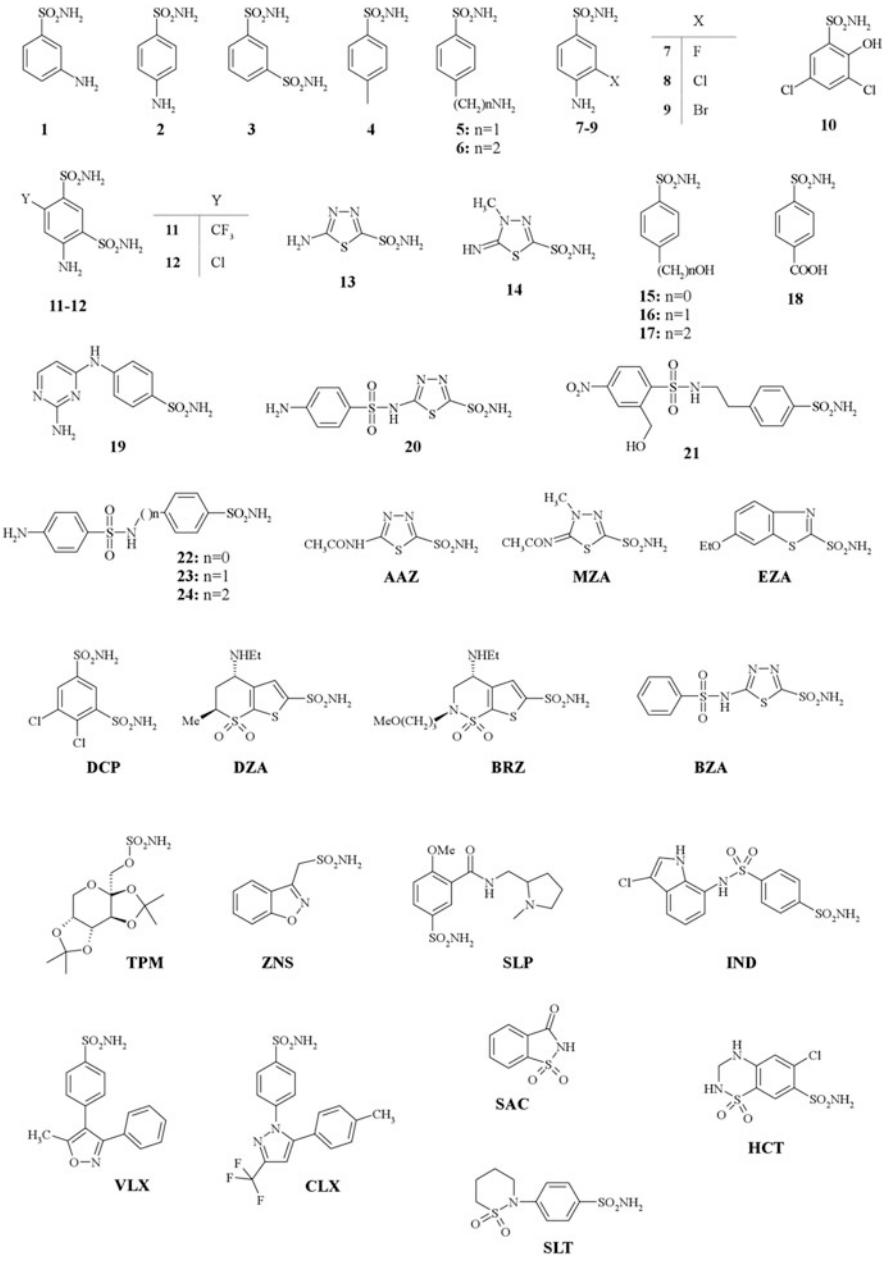


**Fig. 4** Chemical structures of sulfonamides **1–24** and clinically used compounds **AAZ–HCT** tested against TvaCA1 enzyme [48]

**Table 3** Inhibition constants of hCA II and TvaCA1 with sulfonamides **1–24** and the clinically used drugs **AAZ–HCT**, measured by a $CO_2$ hydrase stopped-flow assay[a]

| Inhibitor | $K_I$ (nM) | |
|---|---|---|
| | hCA II | TvaCA1 |
| **1** | 300 | 3,246 |
| **2** | 240 | 4,742 |
| **3** | 8 | 3,559 |
| **4** | 320 | 3,599 |
| **5** | 170 | >50,000 |
| **6** | 160 | >50,000 |
| **7** | 60 | 4,282 |
| **8** | 110 | >50,000 |
| **9** | 40 | >50,000 |
| **10** | 54 | 4,536 |
| **11** | 63 | >50,000 |
| **12** | 75 | >50,000 |
| **13** | 60 | 1889 |
| **14** | 19 | 3,987 |
| **15** | 80 | 2027 |
| **16** | 94 | >50,000 |
| **17** | 125 | >50,000 |
| **18** | 46 | >50,000 |
| **19** | 33 | 4,528 |
| **20** | 2 | >50,000 |
| **21** | 11 | 3,450 |
| **22** | 46 | >50,000 |
| **23** | 33 | >50,000 |
| **24** | 30 | >50,000 |
| **AAZ** | 12 | 391 |
| **MZA** | 14 | 3,827 |
| **EZA** | 8 | 283 |
| **DCP** | 38 | >50,000 |
| **DZA** | 9 | >50,000 |
| **BRZ** | 3 | >50,000 |
| **BZA** | 9 | >50,000 |
| **TPM** | 10 | >50,000 |
| **ZNS** | 35 | >50,000 |
| **SLP** | 40 | >50,000 |
| **IND** | 15 | >50,000 |
| **VLX** | 43 | >50,000 |
| **CLX** | 21 | >50,000 |
| **SLT** | 9 | >50,000 |
| **SAC** | 5,959 | >50,000 |
| **HCT** | 290 | >50,000 |

[a]From reference Urbanski et al. [48]

human enzyme was significantly better inhibited. These studies clearly indicate that sulfonamide molecules generally behave as better inhibitors of human isoforms with respect to TvaCA1, thus do not represent ideal lead compounds for the development of selective TvaCA1 inhibitors [48].

## 4 Conclusions and Future Perspectives

The diagnosis and effective treatment of *T. vaginalis* infection has become an extremely important goal for global health in both women and men, due to the increasing experimental evidences showing the relationship between trichomoniasis and other critical pathologies and the appearance of resistance to the existing pharmacological treatments. Consequently, in recent years research of novel drug targets for fighting trichomoniasis has seen an increased interest. In this scenario and considering recent experimental evidences, which indicate CAs as potential targets for the treatment of protozoan parasitic diseases, our group focused the attention on TvaCA1, a β-CA from *T. vaginalis*, carrying out a complete biochemical, structural, and kinetic characterization of this enzyme. The enzyme was demonstrated to possess a rather high catalytic efficiency and to behave as a non-covalent dimer in solution. The crystal structure determination highlighted significant differences between the active site of TvaCA1 and that of human CAs. Moreover, the parasitic enzyme could be inhibited both by sulfonamides in the nanomolar range and by other small molecules such as phenylboronic and phenylarsonic acid in the micromolar range. The latter two compounds, although being less efficient than sulfonamides, emerged as ideal lead compounds for the development of anti-trichomoniasis drugs, since they were rather selective for TvaCA1 with respect to hCA I and hCA II.

**Compliance with Ethical Standards** *Conflicts of interest*: The authors declare no competing interest.

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*Ethical approval*: This work is a review of previously published accounts, as such, no animal or human studies were performed.

*Informed Consent*: No patients were studied in this chapter.

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# Treatment of Toxoplasmosis: An Insight on Epigenetic Drugs


**Paolo Guglielmi and Daniela Secci**

## Contents



**Abstract** Toxoplasmosis is the parasitic infection caused by the obligate intracellular parasite *T. gondii*. This pathogen possesses three different stages of life, namely (1) sporozoites, (2) tachyzoites (3) and bradyzoites, the slow replicating form living in tissue cysts. To date, the clinical therapy of toxoplasmosis is still based on the use of drugs developed more than 50 years ago and endowed with high toxicity and ineffectiveness against bradyzoites, preventing the complete eradication of the parasite. For these reasons, novel and more effective drugs are still necessary. Epigenetics drugs could fulfil this requirement offering novel mechanisms of action also affecting the bradyzoite stage. Here we report the inhibitors of *T. gondii* affecting epigenetic targets discovered in the last 25 years.

**Keywords** Cyclic polypeptides, Epigenetic regulation, Hydroxamates, Tachyzoites-bradyzoites conversion, TgGCN5 inhibitors, TgHDAC inhibitors, *Toxoplasma gondii*

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## 1 Introduction

*Toxoplasma gondii*, a member of the Apicomplexa phylum, is an obligate intracellular parasite and the causative agent of toxoplasmosis. This parasite was reported for the first time in the 1908 by Nicolle and Manceaux, from the North African rodent *Ctenodactylus gundi* and in the same year, Splendore (Brazil) found this microorganism in rabbits [1, 2]. Toxoplasmosis is considered as a tricky infection due to the multistage life cycle of *T. gondii* that involves different hosts: cats and other Felidae serve as definitive hosts and are the only species where *T. gondii* can sexually reproduce [3], whereas warm-blooded mammals like humans are intermediate hosts [4, 5]. The life cycle of *T. gondii* involves sexual and asexual reproductive phases [2]. In the epithelial cells of the cats' small intestine, schizogony (asexual reproduction) and gametogony (sexual reproduction) occur, prompting the production of unsporulated oocysts that are then shed in their faecal matter [6]. Felines are the only mammals that lack delta-6-desaturase activity, required for linoleic acid metabolism, in their intestines which results in systemic excess of linoleic acid. This condition seems to act as a positive signal for *T. gondii* sexual development [7]. During its life, *Toxoplasma gondii* can pass through 3 cycle stages:

1. oocyst, containing sporozoites, with a multilayer structure that protects the organism from chemical and mechanical changes.
2. Tachyzoite, the highly proliferative form of parasitic life, that can disseminate to multiple and distant tissues within the host's body. Tachyzoites are present during the acute infections; they establish themselves within a parasitophorous vacuole in which they multiply (replicate every 6–8 h in a process known as "endodyogeny") and then actively egress to invade neighbouring cells [5]. This is called lytic cycle and when repeated multiple times, will cause considerable tissue damage and is responsible for the symptoms of the acute phase of the disease [8].
3. Bradyzoite, the slower replicating form, differentiates from the tachyzoite stage and is prevalent in chronic infections [9]. Indeed, in the presence of stress conditions as well as intermediate hosts immunocompetent response, tachyzoites transit to the latent bradyzoite form. Bradyzoites are confined inside tissue cysts, located in the brain and muscle of most of the intermediate hosts. Albeit cysts usually remain dormant in immunocompetent patients, in the retina they frequently reactivate leading to recurrent chorioretinitis [10].

Cats can contract toxoplasmosis through the ingestion of bradyzoites, tachyzoites or oocysts coming from contaminated prey such as birds or rodents. In human beings, toxoplasmosis is reported to be transmitted via undercooked meat infested with latent cysts, contaminated water with sporulated oocysts and ingested contaminated food [11]. After ingestion of the cyst containing bradyzoites, the wall of the cyst dissolves during digestion and the bradyzoites are released. This stage of the parasite is highly resistant to protease activity and easily survives in the small intestine of the host, initiating infection [12]. Ingestion of oocyst from contaminated

food or water led to more severe infections if compared with tissue cyst-acquired ones. Further routes of transmission of *Toxoplasma gondii* between human hosts are through semen, if the secondary host is male, and through congenital transmission, if the secondary host is a pregnant female [13–16]. The congenital transmission happens when toxoplasmosis is at the acute phase and tachyzoites coming from the mother cross the placenta causing infection in the foetus [17]. The risk and severity of symptoms in congenital infection mainly depend on the age of gestation and on the stage of pregnancy. When the mother gets infected between week 10–24 of pregnancy, the risk for severe defects in the new-born is about 5–6% [3]. The impacts of toxoplasmosis differ depending on the parasite's characteristic features such as inoculums size, the virulence of the infecting strain, host factors such as immune status and genetic background of the individuals [18]. In most individuals with competent immune responses, primary infection is asymptomatic or may produce a mild, flu-like illness [5]: in these individuals, tachyzoites are eliminated by the immune system. After the primary infection, the host will develop several antiparasitic mechanisms that first include innate immunity but also adaptive and cell-autonomous responses. However, the parasite has evolved strategies to successfully bypass or manipulate the immune system by secreting proteins that modify host transcriptional programs or signalling pathways [10]. On the contrary, in immunocompromised individuals, such as people affected by HIV, patients who received haematopoietic stem cells or a solid organ transplant, *T. gondii* can cause severe illness and/or death [9]. The disease's transmission varied widely among populations, mainly based on food habits and culture. Despite its asymptomatic nature, it necessitates the recommendation of various effective steps for the management, diagnosis, and prevention of the disease. In fact, without therapeutic interventions or a strong immune response, *T. gondii* will cause severe or even fatal pathologies.

## 2 Toxoplasmosis Treatment

In the wake of the finding in 1942 of Sabin and Warren about the ability of sulfonamides to act as anti-*T. gondii* drugs [19], efforts have been done in order to develop even more active compounds able to eradicate this parasite infection. After nearly 10 years by this discovery, the synergistic effect coming from the combination of pyrimethamine-sulfadiazine against experimental toxoplasmosis in mice was discovered [20]. Pyrimethamine-sulfadiazine coadministration is still the gold standard, the novel regimens being compared with it. It takes advantage of the combination of two antimicrobials, pyrimethamine and sulfadiazine, working as inhibitors of dihydrofolate reductase (DHFR) and dihydropteroate synthetase, respectively [21–23]. Other compounds endowed with these properties are trimethoprim targeting DHFR, and additional sulfonamides such as sulfadiazine, sulfamethoxazole, and sulfadoxine, blocking dihydropteroate synthetase. These enzymes are responsible for the folate synthesis, taking part in two successive steps of the folic

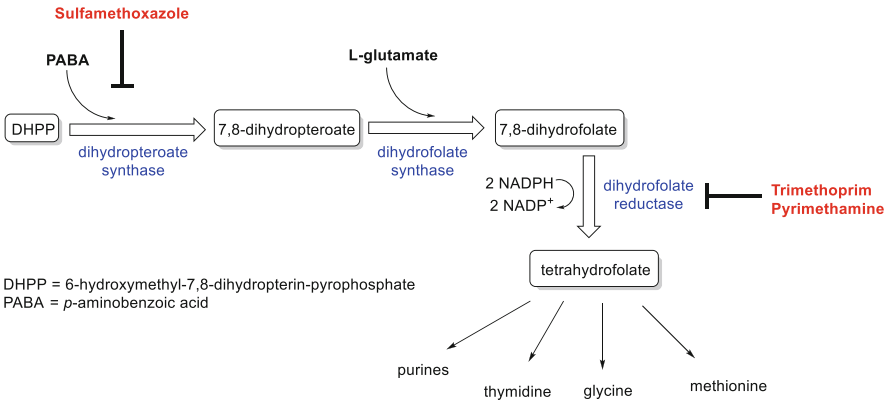


**Fig. 1** Folic acid biosynthesis pathway and inhibitory activity of pyrimethamine and/or trimethoprim and sulfonamides

acid biosynthesis (Fig. 1) [24, 25]. The contemporary blockage of the same biosynthetic pathway at two different stages led to impressive increase of effectiveness and at the well-known synergistic effect [23].

In the years other combinations have also been discovered and employed in the clinical practice. The treatment of toxoplasmosis is tailored considering the immune system condition of the patients, differing in the dosages of the drugs. In immunocompetent patients as well as immunocompromised ones, the first choice is pyrimethamine-sulfadiazine combination. Other regiments include trimethoprim-sulfamethoxazole coadministration or, in the presence of patient with intolerance to sulpha drugs, the combination of pyrimethamine with clindamycin or azithromycin, both inhibitors of the bacterial 50S ribosomal subunit. These drugs probably act on the apicoplast of the parasite, a plastid organelle where take place several essential metabolic pathways [26–32]. These combinations (pyrimethamine-clindamycin or pyrimethamine-azithromycin) are often used for patients with AIDS [33–35]. Recently, the combination of clindamycin and azithromycin has been explored as alternative treatment for *Toxoplasma gondii* encephalitis in a 57-year-old HIV-positive man intolerant to trimethoprim/sulfamethoxazole, pyrimethamine, and sulfadiazine [36]. Another association is pyrimethamine-atovaquone, the last one being a mitochondrial electron transport inhibitor in the malarial parasite [37] (Fig. 2). Interestingly, due to the absence of toxicity and its inability to cross the placenta, spiramycin, a macrolide antibiotic discovered in the 1958, is still used as preventive treatment in pregnant women to avoid materno-foetal transmission of the parasite [4, 29, 38, 39]. Apart from spiramycin, all these combinations require the contemporary administration of folic acid; furthermore, they suffer of similar toxicity requiring continuing monitoring (blood counts, creatinine, and liver function) and proper patients' behaviour (adequate hydration should be ensured to prevent renal damage from crystalluria induced by sulfonamides). Another aspect limiting the effectiveness of these compounds is their ability to exclusively affect tachyzoite

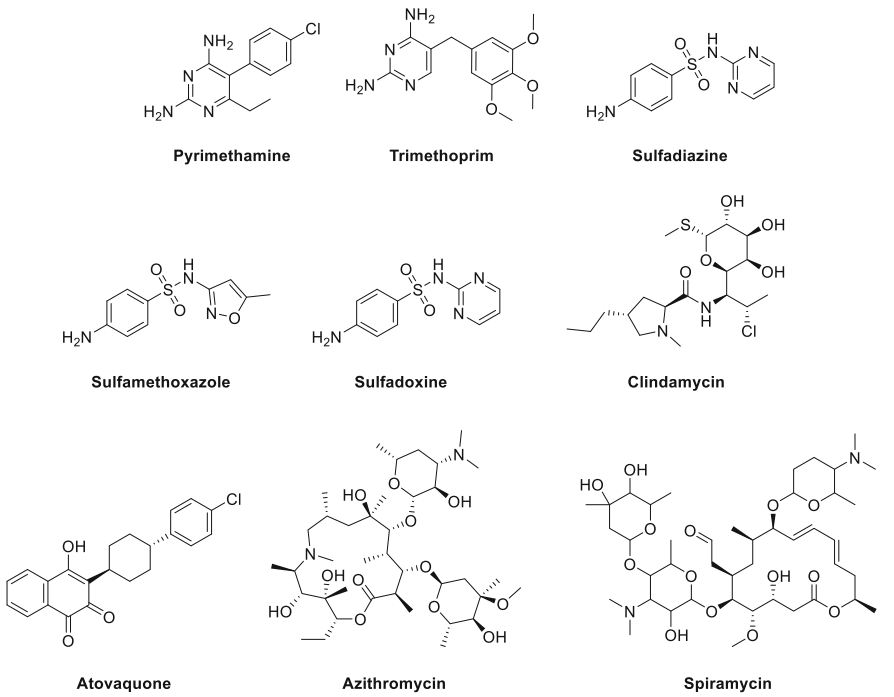


**Fig. 2** Structure of the drugs currently used to treat Toxoplasmosis

stage of the parasite, being inactive against cysts containing bradyzoites, the latent stage of the parasite. So, the development of novel drugs endowed with less toxicity and more effectiveness is still required.

## 3 *T. Gondii* Inhibitors: An Overview

The early research about anti-*Toxoplasma* compounds dates back to 1940. In the last 80 years, a huge number of works focused on novel drugs discovery for *T. gondii* treatment [40–46]. These efforts contributed mostly to the development of novel chemical libraries, as well as the discovery of novel molecular targets useful to obtain innovative strategies to fight the parasite. In a recently reported review Deng and colleagues deeply and extensively described the last research about anti-*T. gondii* derivatives, grouping the compounds in different categories [46]. To avoid redundant analysis of the same structures and inhibitors, we briefly described the various categories of *T. gondii* inhibitors (for major details about the other classes of inhibitors we recommend the references [44, 46]), focusing our efforts on the research performed in the last 25 years about *T. gondii* inhibitors acting on epigenetic targets.

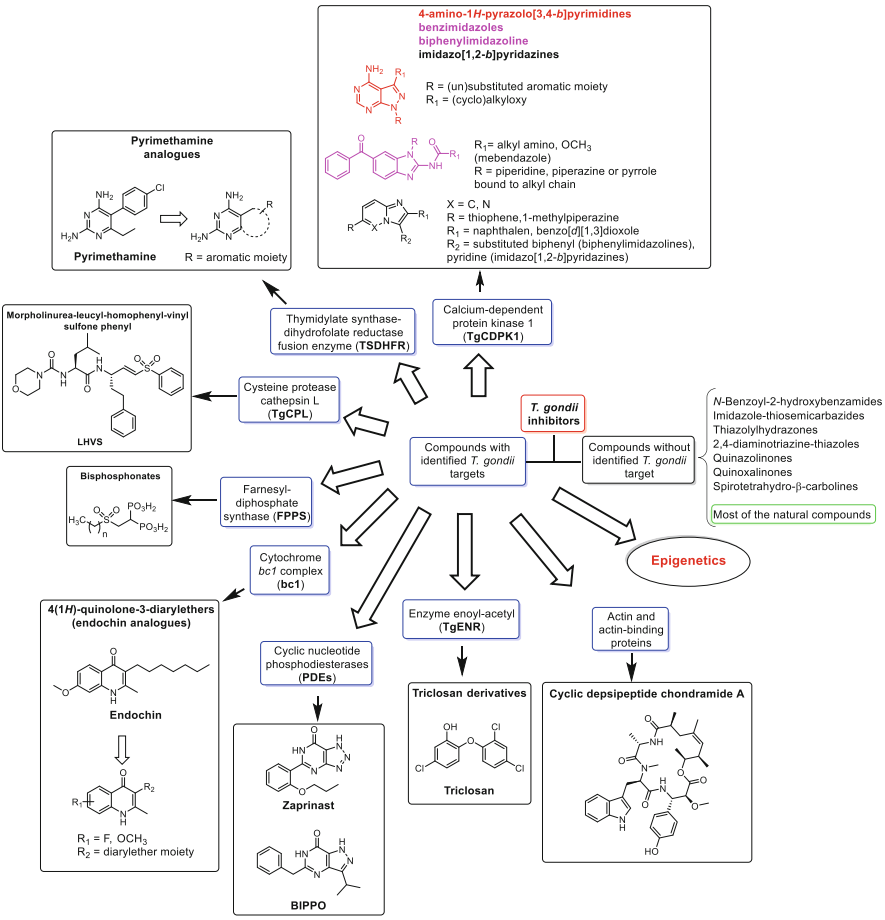


**Fig. 3** Inhibitors of *T. gondii* with known and unknown targets

Concerning *T. gondii* inhibitors, it is possible to collect them in two main groups: (1) compounds with unknown targets (2) and compounds with known targets (Fig. 3). The first group includes anti-*T. gondii* compounds whose mechanism of action is not sill understood, requiring deepening in order to shed light on the parasite's molecular/enzymatic targets. Different scaffolds such as *N*-benzoyl-2-hydroxybenzamides, thiazolylhydrazones, 2,4-diaminotriazine-thiazoles and most of the natural compounds, belong to this category [46].

The second group comprises synthetic small molecules whose enzymatic/cellular targets have been identified. One of the mainly investigated is calcium dependent protein kinase-1 (TgCDPK-1), a crucial enzyme of *T. gondii,* playing a pivotal role in several processes that are critical to the intracellular replicative cycle of the parasite [47–51]. Due to the presence of an "atypical" ATP-binding pocket endowed

with a glycine residue in spite of larger hydrophobic ones, TgCDPK-1 is sensitive to ATP competitive inhibitors able to accommodate bulky hydrophobic groups in this expanded pocket. Because glycine gatekeeper is unprecedented in human kinases, this attribute can be exploited to obtain selective drugs. The main scaffolds employed to develop TgCDPK-1 inhibitors are 4-amino-1*H*-pyrazolo[3,4-*b*]pyrimidines, benzimidazoles, biphenylimidazoline and imidazo[1,2-*b*]pyridazines [30, 46] (Fig. 3).

Similar to other protozoal parasites *T. gondii* has a bifunctional dihydrofolate reductase–thymidylate synthase (DHFR-TS) containing on the same protein both the catalytic sites [52, 53]. Pyrimethamine (Fig. 1), one of the first-line drugs currently used to treat toxoplasmosis, targets this enzyme. However, due to the interference with the host folate metabolism, pyrimethamine is always administered along with folic acid supplementation. Furthermore, its teratogenicity prevents its use during pregnancy. However, inhibitors exhibiting greater selectivity for *T. gondii* DHFR could potentially be less toxic. In general, inhibitors that act against this target are based on, or are mainly developed from, pyrimethamine scaffold [54–56]. Other enzymes like the cysteine protease cathepsin L (TgCPL) and the farnesyl-diphosphate synthase (FPPS) have been recognized as exploiting targets for the development of anti-*T. gondii* compounds [57–60]. The first, TgCPL, is critical for the chronic infection of *T. gondii*; indeed, bradyzoites missing of TgCPL die after forming cysts in both culture and in the neuronal cells of infected mice [61]. One of the most representative inhibitors of this target is morpholinurea-leucyl-homophenyl-vinyl sulfone phenyl (LHVS, Fig. 3) [57]. The second, FPPS, is a key enzyme for the biosynthesis of isoprenoid, catalysing the consecutive condensations to form farnesyl diphosphate, a substrate used for the production of sterols, dolichols, heme A, ubiquinone, and prenylated proteins. It is preferentially inhibited by bisphosphonates, metabolically stable pyrophosphate analogues containing a $CH_2$ in spite of the oxygen atom bridge between the two phosphorus atoms of the pyrophosphate [59–63].

The cytochrome *bc1* complex (*bc1,* ubiquinol:cytochrome c oxidoreductase), a constituent of the mitochondrial electron transport chain, is the target of Atovaquone one of the drugs used in association with pyrimethamine (as an alternative treatment), and endochin-like quinolones [64–66]. Triclosan and its analogues are able to inhibit the enzyme enoyl-acetyl (TgENR) participating in the type II fatty acid synthesis (FAS II) pathway, that supplies the necessary fatty acid for the growth of parasite [67, 68]. Cyclic nucleotide phosphodiesterases (PDEs) control downstream process in the parasite cell by regulating the concentration of the 2 s messengers cyclic-AMP (cAMP), cyclic-GMP (cGMP), that in turn affect kinases activity influencing cellular program. Two inhibitors able to affect these enzymes are zaprinast and BIPPO [46]. Among the *T. gondii* inhibitors endowed with known mechanism of action there are epigenetic drugs (i.e. compounds acting on epigenetic targets) that will be deepened in the following paragraphs.

## 4 Epigenetics: A New Opportunity for *T. Gondii* Inhibitors?

The first evidence about the opportunity to use epigenetic approaches for the development of *T. gondii* inhibitors dates back to 1996, when Darkin-Rattray and co-workers discovered the ability of a series of natural cyclic tetrapeptides, inhibitors of human histone deacetylase (HDAC), to exert antiproliferative effects against some parasites, including *T. gondii* [69]. This work claimed that enzymes implicated in chromatin modification may be exploited to obtain effective new therapies against this pathogen [70]. Albeit the fundamental principles of epigenetic regulation in *T. gondii* are quite similar to those taking place in mammalian cells and model systems, it also displays some exclusive mechanisms. The term epigenetics is used to describe all those mechanisms contributing to the phenotype without changing genome sequence but influencing the degree of gene expression. Indeed, by changing gene expression levels, the same genome can potentially produce a wide variety of phenotypes. The main routes that cells use to affect gene expression include DNA methylation, nucleosomal remodeling, and covalent modification of histones, the last one being deeply investigated since 1996, after the discovery of a histone acetyltransferase (HAT) homologous of the yeast transcriptional adaptor called GCN5 (general control nonderepressible-5) in *Tetrahymena* [70]. In 1999 there was the discovery of a *T. gondii* HAT homologue of GN5 (Tg-GN5) and 6 years after two more HATs belonging to the MYST family (TgMYST-A and -B) were characterized [71–73]. Due to the ability of these enzymes to act also on other targets over the histones, they are properly named lysine acetyltransferases (KATs). These enzymes belong to the category of the so-called writers because they mark chromatin. For example, KATs catalyse the acetyl group transfer from its substrate, acetyl-CoA, to the target proteins at the selected ϵ-amino groups of a lysine residue. The counterparts of the "writers", i.e. enzymes removing the marks, are called "erasers". An example of erasers are the histone deacetylases HDACs (also termed lysine demethylases KDACs for the same reasons seen before for the KATs). These enzymes catalyse the removal of the acetyl group, thus restoring ϵ-amino groups of lysine residues and were described for the first time in *T. gondii* along with histone arginine methyltransferases by Saksouk et al. (Fig. 4) [74]. There are also proteins called "readers", endowed with domains able to recognize and interpret these post translational modifications (PTMs). These sites are called bromodomains (BRDs) if recognized acetylated lysine, while there are other sites able to distinguish methylated lysines or arginines (e.g., chromodomains, PHD fingers and Tudor domains) [75].

One of the main substrates of these enzymes are histones, nuclear basic proteins organized in way to form an octamer composed of two copies of each of the four histones: H2A, H2B, H3 and H4. The histone octamer is wrapped twice by 147 bp of DNA forming a structure representing the fundamental repeating unit of chromatin, the nucleosome. This structure is stabilized by the "linker" histone H1. Albeit this general scheme is quite conserved, several variant histones have been discovered in Eukaryotes and may be used in place of the canonical ones. As an example, *T. gondii*

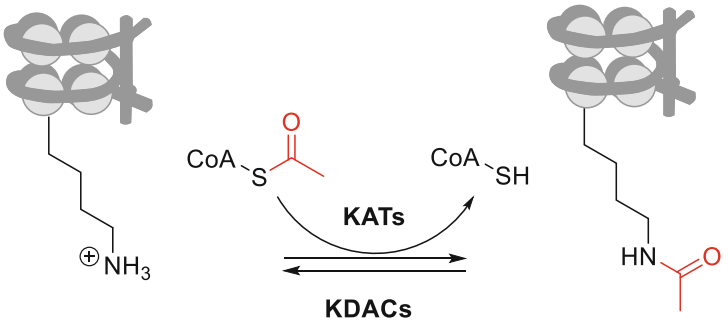


**Fig. 4** Activity of KATs and KDACs

is devoid of the H1 histone, while is able to encode the variants H3.3 and CenH3 of H3, the H2A.Z and H2A.X, variants of H2A and a parasite-specific H2B variant (H2Bv, now renamed H2B.Z) [76]. Histone modifications through acetylation, methylation, phosphorylation, glycosylation, ADP-ribosylation, ubiquitination and SUMOylation affect chromatin affinity for macromolecular complexes. Indeed, these changes can alter the accessibility to transcriptional complexes and polymerases, in a process of "activation" or "de-activation" of gene expression due to the state of chromatin: euchromatin (loose, accessible) or heterochromatin (compact, inaccessible). For example, acetylation of lysine residues placed on histone proteins neutralizes the positive charges, weakening the interactions with negatively charged DNA leading to loose chromatin [77]. These changes producing specific downstream effects paved the way for "the histone code" hypothesis [78]. Another strategy useful to modify the condensation state of chromatin is the DNA methylation performed by DNA methyltransferase enzymes (DNMT); however, this mechanism does not appear as the one preferred to control *T. gondii* gene expression. All the epigenetic inhibitors of *T. gondii* explored so far possess Tg-HDAC or TgGCN5 inhibitory activity (see below), these two classes of enzymes being mainly investigated.

Up to date 18 HDACs have been identified in eukaryotes and depending on sequence similarity and cofactor requirement have been grouped in four classes (I-IV) [79]. Mammalian class I HDACs are localized in the nucleus and include HDACs 1, 2, 3 and 8; class II HDACs, shuttling between the nucleus and cytoplasm in dependence of the tissues, contain the HDACs 4–7 and 9–10. The class IV contains only one member (HDAC11), while the class III (also called sirtuins, SIRTs) differed from all the other classes being $NAD^+$-dependent enzymes (SIRT1–7). Indeed, the other HDACs share a common catalytic core exploiting $Zn^{2+}$ as cofactor, albeit differing in size and structural organization [80]. In *T. gondii* five type I HDAC homologues (TgHDAC1–5) have been discovered [81]. These enzymes are essential for tachyzoite proliferation and are probably implicated, along with acetylases, in the tachyzoite-bradyzoite gene regulation [74]. The most investigated and characterized *T. gondii* HDAC is Tg-HDAC3, a nuclear protein sharing

60% of sequence identity with hHDAC1 and taking part in a large multiprotein complex termed *T. gondii* corepressor complex. Also type III HDACs (SIR2 and SIR2b) have been observed, but similar to that observed in *P. falciparum*, seem to be unessential [82].

Considering the homology to yeast orthologues as well as catalysis mechanism, three major families have been proposed for KAT enzymes: the p300/CREB-binding proteins (p300/CBP), the GCN5-related *N*-acetyltransferases (GNAT), and the MOZ, Ybf2, Sas2, and Tip60 (MYST) family [77]. As previously assessed, *T. gondii* possesses two KATs belonging to the MYST family, MYST-A and MYST-B; they are endowed with histone H4 acetylation activity and MYST-B appeared to be implicated in parasite replication rate regulation [73]. *T. gondii* possesses two histones acetyltransferases orthologous of GCN5 [83] that act modifying H3 and probably H4 and playing a pivotal role in parasite proliferation as well as bradyzoite differentiation. The two proteins are described as the essential TgGCN5-B and unnecessary TgGCN5-A, because its removal did not alter phenotype in tissue culture [83–85]. However, TgGCN5-A seems to be involved in stress response, controlling the expression of the stress-induced genes, and life cycle progression [86]. On the other hand, the induction of a dominant negative version of TgGCN5-B containing an inactive KAT domain led to dysregulated gene expression and arrested replication [84]. Further mechanisms involved in epigenetic regulation are associated with protein methylation-demethylation performed by protein arginine methyltransferases (PRMTs) and lysine methyltransferases (KMTs). However, *T. gondii* inhibitors targeting these enzymes have not been discovered, yet. For major details about these enzymes and their role in *T. gondii* gene regulation we recommend the work of Kim [81].

## 5 Epigenetic Drugs for the Treatment of Toxoplasmosis

The compounds reported as *T. gondii* inhibitors based on epigenetic mechanisms can be considered as repurposed drugs, most of them being investigated for antitumoural intent in human beings. However, this approach can be useful to define a series of lead compounds that can be successively modified in order to increase selectivity against parasite cells in spite of the host ones.

### *5.1 Cyclic Tetrapeptide HDAC Inhibitors*

As assessed above, the first evidence about the opportunity to employ epigenetic drugs as anti-*Toxoplasma gondii* agents came from the study performed by Darkin-Rattray and colleagues that evaluated a series of natural cyclic tetrapeptides endowed with human histone deacetylase (HDAC) inhibitory activity, against a series of Apicomplexa parasites including *T. gondii* [69]. Among them, Apicidin, Apicidin

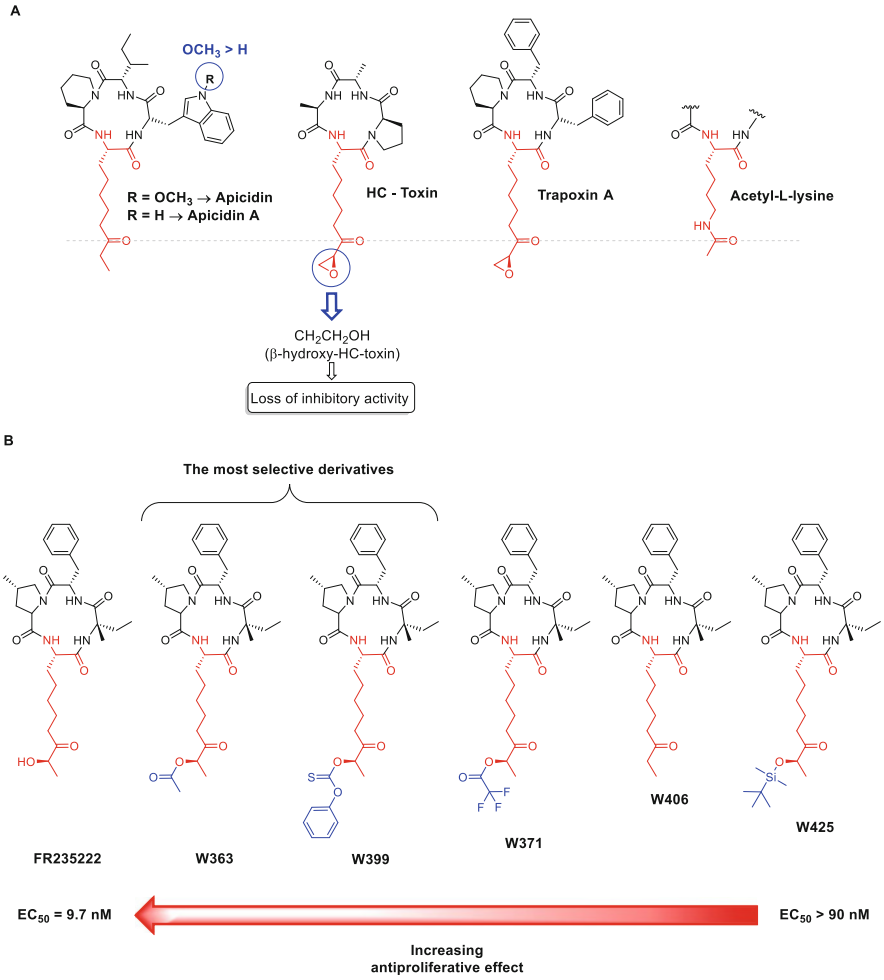


**Fig. 5** (**a**) Structure of tetrapeptides Apicidin A, Apicidin, HC-Toxin, β-hydroxy HC-Toxin and Trapoxin A. (**b**) Structure and activity of FR235222 and related analogues

A and HC-Toxin displayed in in vitro tests a minimal inhibitory concentration (MIC) at the nanomolar range against the tachyzoite stage of *Toxoplasma gondii* (12.8–34.3 nM), differing from β-hydroxy-HC-Toxin that did not exhibit antiproliferative activity (Fig. 5a).

Apicidin was the most effective compound (MIC = 12.8 nM) while the removal of the N-bound methoxy group to afford Apicidin A led to a slight reduction of the antiproliferative activity at value similar to that observed for HC-Toxin (MIC = 25.3 nM). The antiproliferative effect of the HC-Toxin was subjected to the presence of the epoxy moiety; indeed, the removal of this molecular attribute by epoxide ring opening to generate β-hydroxy HC-Toxin led to inactive compound

(MIC >1,000 ng/mL). All these molecules, along with another well-known mammalian HDAC inhibitor (HDACi) Trapoxin A, share (over the cyclic tetrapeptide motif) a side chain that mimics the acetyl-L-lysine, the substrate of the HDAC enzymes (shown in red in the Fig. 5) with the carbonyl groups of the inhibitors being isosteric to the scissile carbonyl amide bond. However, these inhibitors differ in addition to the amino acids constituting the cyclic tetrapeptide, also for the final part of the chain accounting for reversible or irreversible inhibition. Apicidin, bearing an ethyl moiety bound to the carbonyl group is endowed with reversible inhibitory activity; on the contrary, the epoxide moiety exhibited by HC-Toxin and Trapoxin A is thought to serve for irreversible inhibitory activity towards class I HDACs [87], albeit recently crystallographic insights on HDAC8 displayed that this moiety did not react with the enzyme, being intact in the crystal structure of its complex with HDAC8 [88]. Indeed, the ketone carbonyl group of Trapoxin A side chain underwent nucleophilic attack by zinc-bound water, similarly to that observed for HC-toxin in HDAC6 [89], leading to a gem-diolate that binds the $Zn^{2+}$ ion, resembling the tetrahedral intermediate of the transition state [88]. This strong interaction along with a favourable staggered conformation of the intact epoxide led to a very tight bond and to a non-covalent irreversible inhibition. These compounds displayed the ability to inhibit Apicomplexa HDAC (HDAC extracted from *E. tenella*) in the nanomolar range, leading to hyperacetylated histones and displaying this enzyme as putative target for the anti-*T. gondii* activity. Further studies involving cyclic polypeptides were performed by Bougdour and co-workers, that examined the effects of FR235222, isolated from the fermentation broth of *Acremonium* species, along with a series of well-known hHDAC inhibitors like trichostatin A (TCA) and scriptaid (Figs. 5b and 6) [90, 91]. FR235222 exhibited interesting properties being able to interfere with the growth of different strains of *T. gondii* tachyzoites infecting human foreskin fibroblast: the RH strain (type I, that is not able to complete the two-host life cycle, but is rapidly replicating), the Prugniaud strain (type II, able to differentiate into bradyzoites) and the CTG strain (type III) were inhibited at low nanomolar levels ($EC_{50} < 15$ nM). In this study also the cyclic polypeptides Apicidin and HC-Toxin (Fig. 5a) were investigated displaying similar potency against the RH strain and confirming the evidences reported by Darkin-Rattray and colleagues [69]; on the contrary, the well-known inhibitors of mammals HDAC, trichostatin A (TCA) and scriptaid (Fig. 6), showed lower inhibition of the *T. gondii* proliferation.

Interestingly, TCA and FR235222 were evaluated against purified Tg-HDAC3 exhibiting similar inhibitory activity. So, the disagreements observed in in vitro tests involving infected cells have to be searched in the differences of physical-chemical properties of the compounds that could affect the ability to cross the host cells and parasitic membranes. Intriguingly, intracellular parasites treated with FR235222 exhibited vacuolation and lacked the inner membrane complex protein 1 (IMC1), a feature of apicomplexan parasites that plays a central role for both their motility and cell division (endodyogeny) [92]. Furthermore, also DNA over-replication was observed, accounting for direct or indirect interference with cell-cycle progression. Histone H4 exhibited increased acetylation (while the H3 was not influenced)

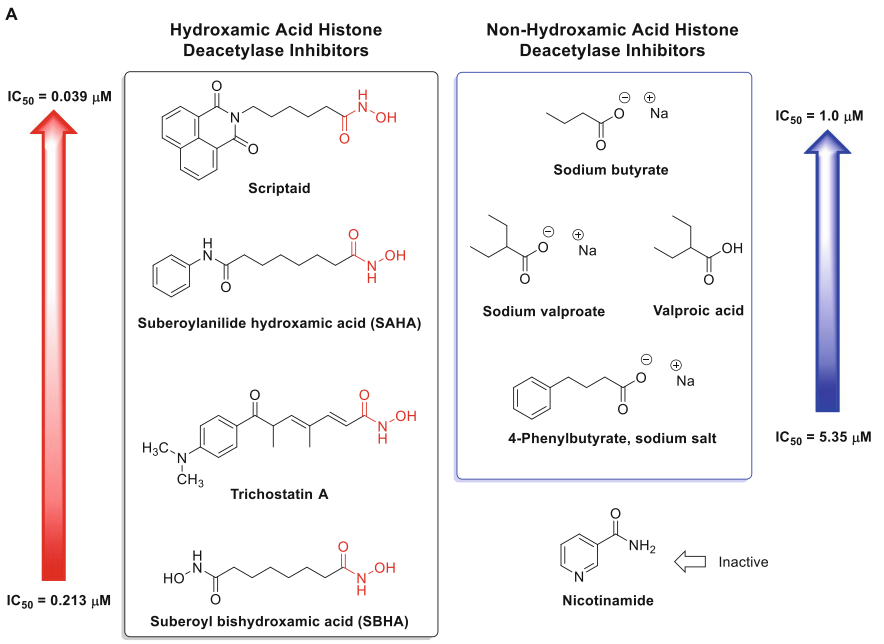


**Fig. 6** Structures of some common mammalian HDAC inhibitors evaluated against tachyzoites of *T. gondii* (RH strain)

probably through the inhibition of the Tg-HDAC3, a nuclear deacetylase whose mutation led to resistance against FR235222 [90]. Indeed, point mutation of the wild type Tg-HDAC3 at level of the T99 residue, taking part of a two-residue extension specific for apicomplexan parasites (A98T99), generated two clones (T99A and T99I) endowed with resistance towards FR235222 treatment. Through the inhibition of Tg-HDAC3, FR235222 influenced the expression of at least 370 genes, affecting the differentiation of the tachyzoite (replicative) into the bradyzoite (non-replicative) stage. The same research group also performed further in vitro, ex vivo and in vivo experiments in order to deepen the effects of FR235222 on the cystic form of *T. gondii* [91]. FR235222 affected the bradyzoite differentiated parasites dramatically altering the morphology of the treated cells with the appearance of giant and multinucleate cells. Albeit ex vivo experiments achieved on cysts isolated from chronically infected mice, evidenced poor outcomes after 7-day treatment with FR235222 the external structure of the cysts being intact, the same cells subjected to the proliferation assay showed the lack of ability to produce bradyzoite-tachyzoite conversion. This behaviour, differing from the ones shown by the cysts treated with DMSO or pyrimethamine that resulted in tachyzoite-induced lysis of the plaque, was addressed to HDACi activity, and was also observed in in vivo models after the inoculation of cysts treated with FR235222. As a matter of the fact, mice inoculated with HDACi-treated cysts did not develop toxoplasmosis. So, FR235222 appeared

to be an inhibitor of the bradyzoite-tachyzoite conversion. FR235222 displayed selectivity towards *T. gondii* being the parasite ~10 times more sensitive than human foreskin fibroblast (HFF) cells, although hyperacetylation of H4 in human cells was observed at high concentration level (1 μM). In order to improve the selectivity index (i.e., SI = $EC_{50}$ RH strain/$EC_{50}$ HFF cells) a series of semisynthetic analogues of FR235222 have been designed (Fig. 5b). In particular, these derivatives were modified at the alkyl chain level, the moiety resembling the acetylated-L-lysine, by functionalization or removal of the α-hydroxyl group (Fig. 5b). Acetylation of the hydroxyl group led to the most active and selective compound among the analogues (**W363**, $EC_{50}$ RH strain = 10.2 nM), being endowed with similar antiproliferative activity of FR235222 but with reduced effect on HFF cells ($EC_{50}$ HFF cells = 632.1 nM, SI = 62). The other most selective compound was **W399** endowed with *O*-phenyl carbonothioate, that exhibited an equal $EC_{50}$ towards parasite cells along with reduced activity against HFF cells, displaying selectivity index of 47.7.

## 5.2 *Hydroxamate-Based and Short-Chain Fatty Acid HDAC Inhibitors*

The infection caused by *T. gondii*, especially in the latent and chronic phase when the parasite localizes within tissue cysts and mainly in the central nervous system, has been associated with a vast array of neuropsychiatric symptoms [93–95]. In the light of the above, Jones-Brando and colleagues evaluated the anti-*T. gondii* activity of a series of commonly used antipsychotics, in the attempt to evaluate the possible correlation between their function as antipsychotics and/or mood stabilizers, with their ability to inhibit the parasite replication [96]. Among the evaluated compounds, valproic acid and its sodium salt form, sodium valproate (Fig. 6), reduced *T. gondii* tachyzoite numbers in vitro with similar median inhibitory dose ($ID_{50\ \text{sodium valproate}}$ = 4.1 μg/mL and $ID_{50\ \text{valproic acid}}$ = 4.5 μg/mL). The therapeutic index of valproic acid was similar to that of the reference drug trimethoprim [97]. Successively, the possibility that the antiproliferative activity versus tachyzoites came from the HDAC inhibition operated by valproic acid was investigated [98]. Strobl and colleagues tested a series of hHDAC inhibitors belonging to the hydroxamic and non-hydroxamic acid categories (Fig. 6) against tachyzoites of *T. gondii* (RH strain) in HS68 infected cells [99]. Among the hydroxamic acid derivatives scriptaid, suberoylanilide hydroxamic acid (SAHA) and Trichostatin A (TSA) exhibited the best anti-*T. gondii* in vitro activity, being endowed with an $IC_{50}$ in the nanomolar range ($0.039 < IC_{50}\ (\mu M) < 0.083$), while suberoyl bishydroxamic acid (SBHA) was the least effective one. However, while scriptaid and SAHA did not demonstrate cytotoxicity against HS68 cells at the concentration of 10 μM, TSA was highly toxic the HS68 cell monolayer being destroyed after 48 h exposition to 1 μM of the drug, and apoptotic cells were already observed after exposure to 500 nM TSA. Scriptaid

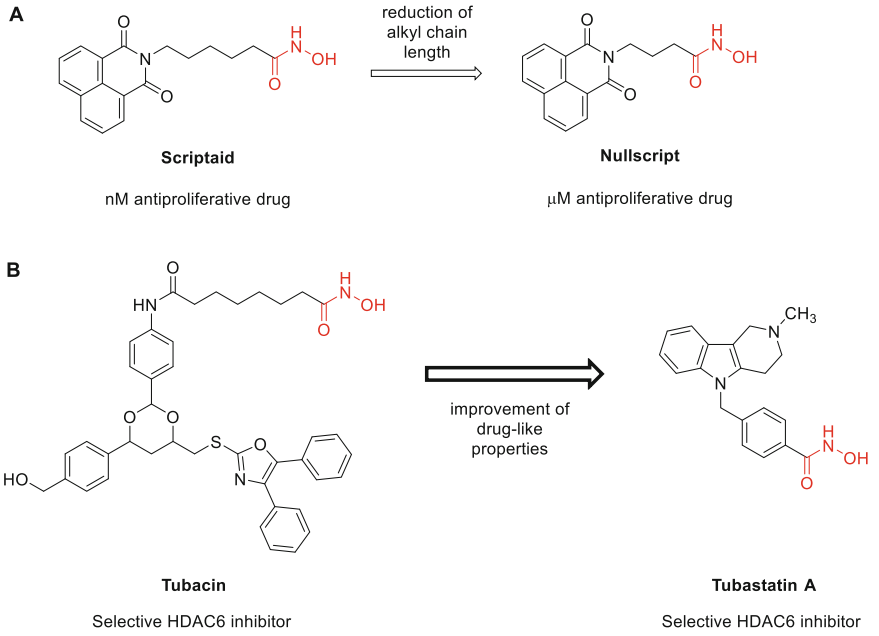


**Fig. 7** Structures of the HDAC inhibitors scriptaid, nullscript, Tubacin and Tubastatin A

and SAHA were more valuable inhibitor of *T. gondii* growth (nM range) than tumour cells in vitro (μM range), indicating that tachyzoites are more sensitive than human tumour cells to these compounds, accounting for selective effect [100–102]. After 48 h exposure of the infected cells to SAHA or scriptaid, the cell HFF monolayer did not exhibit damages; furthermore, 7 days after the treatment no evidence of residual *T. gondii* was detected, accounting for a high effectiveness of these drugs. The non-hydroxamic acid derivatives, sodium butyrate, valproic acid, and 4-phenylbutyrate displayed higher $IC_{50}$ concentration consistent with their reduced potency against HDAC and underlying the importance of the hydroxamic acid moiety (in red in the Fig. 6) as zinc binding group for HDAC inhibition.

The authors assessed as putative targets of these inhibitors the enzyme produced by the RH *T. gondii* gene *hdac3*, sharing 83% nucleotide identity with human *hdac3* active site, albeit also Tg-HDAC5 was also considered.

Reducing the dimensions of the alkyl chain bearing the hydroxamic acid moiety of scriptaid to obtain its analogue nullscript (Fig. 7) was detrimental for the antiproliferative effect, leading to micromolar range inhibitor [103]. Nullscript was evaluated against human foreskin fibroblast (HFF) cells infected with the strain RH-2F of *T. gondii* (expressing β-galactosidase useful for colorimetric assessment of drug activity in vitro as already done by Brando et al. [96, 104]) obtaining an $IC_{50} = 50.9$ μM and underlying the importance of this molecular attribute. Further insights about the effects of SAHA against three strains of *T. gondii* have been recently proposed by Araujo-Silva and colleagues [105]. The authors evaluated the

outcomes coming from the treatment of three different genotypes of *T. gondii*, such as RH (type I), EGS (I/III) and ME49 (type II) strains, with SAHA and another histone deacetylase inhibitors Tubastatin A (TST) whose structure was inspired by Tubacin (Fig. 7) [106]. This compound is selective for mammal HDAC6 and currently is investigated for its beneficial effects in neurological diseases and for its ability to allow the overcoming of multidrug resistance observed in glioblastoma multiforme (GBM) [106–111]. The effects of the two HDAC inhibitors were evaluated against the tachyzoites of the three strains for 24 h and 48 h of treatment, obtaining the inhibition of the *T. gondii* proliferation at the nanomolar levels.

Forty-eight hours treatment of the EGS strain bradyzoites with SAHA and TST significantly inhibited the proliferation/viability of bradyzoites at the nanomolar and micromolar range, respectively. Taking advantage of plaque assay, irreversibility of the effects elicited by treatment of normal human dermal fibroblasts (NDHF) was evaluated, displaying the ability of these drugs to prevent parasite proliferation and protect monolayer integrity, also after drug removal. Cytotoxicity assays performed on rhesus monkey kidney epithelial cells (LLC-MK2), NDHF cells, i.p. (intraperitoneal) macrophages, and primary microglial cultures exhibited safe profile being the concentration required for antiparasitic activity ineffective against host cells, thus accounting for high selectivity index between parasites and host cells. The authors noticed different outcomes coming from the treatment with TST and SAHA spanning from changes of acetylation levels of H3 and H4 (with TST that increased H4 acetylation level while reduced the acetylation of H3) to alteration of cytoskeleton proteins also regulated by acetylation/deacetylation cycles. These data led to the hypothesis that the effects of SAHA and TST should be related to the action against nuclear HDACs, such as Tg-HDAC3, but also against cytoplasmic ones being involved in the PTM of different proteins.

In two different works Loeuillet and colleagues reported the capability of a series of hydroxamates to inhibit parasites in vitro, including *T. gondii* [112, 113]. They assessed the inhibitory activity of a series of aminophenylhydroxamate and aminobenzylhydroxamate derivatives (Fig. 8) against the *T. gondii* Type I RH strain engineered to express the yellow fluorescent protein (YFP) [114], or the Tomato-type II Prugniaud (Pru) strain, infecting HFF monolayer.

Among the evaluated compounds only three demonstrated micromolar/sub-micromolar $IC_{50}$ [112]. The derivatives differed for the presence (or absence) of a methylene group before the nitrogen atom (Fig. 8a), linked to carbonyl or sulfonyl group to obtain amide or sulfonamide, respectively. Furthermore, another methylene group could be present after this moiety as a spacer between the amide/sulfonamide group and the terminal phenyl ring (Fig. 8b).

For compounds bearing 3-fluoro substituted phenyl ring, the presence of the sulfonamide moiety was detrimental for the inhibitory activity, regardless of the presence of the methylene group. On the contrary, by placing the amidic linker in spite of the sulfonamidic one, the $IC_{50}$ dropped to micromolar range only in the presence of methylene spacer before the nitrogen. Similar results were observed with presence of 4-(2-methylthiazol-4-yl) substituted phenyl ring. Interestingly, the most active compound of the series (named **363**, $IC_{50} = 0.35$ μM) was obtained by

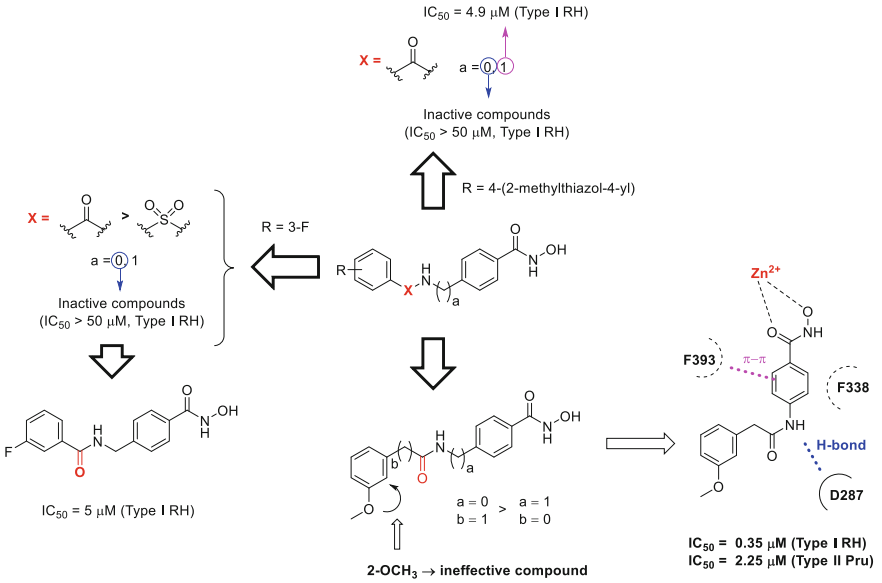


**Fig. 8** SAR of the aminophenylhydroxamate and aminobenzylhydroxamate derivatives

"displacing" the methylene spacer after the amide link and placing a methoxy group at the *meta*-position of the phenyl ring. Interestingly, the simple movement of the methoxy substituent from the *meta* to the *ortho* position led to an impressive loss of activity, probably due to conformational changes moving away the amide group from aspartate residue, thus eliminating hydrogen bond interaction [113]. The compound **363** also exhibited a high selectivity for tachyzoites stage of the parasite, with selectivity indexes of 300 and 10 against HFF and human monocytic cell line THP-1, respectively.

## *5.3 GCN5 Inhibitors*

To date only two works have been reported about the inhibition of *T. gondii* replication obtained through the use of compounds acting on the acetyl transferase GCN5, indicating a more slowly progression of this kind of inhibitors with respect to the HDAC ones [115, 116]. The first evidence about the effectiveness of GCN5 inhibitors for *T. gondii* treatment involved garcinol, a polyisoprenylated benzophenone isolated from *Garcinia indica* (Fig. 9) [115]. Garcinol exhibited in vitro antiproliferative effects against Type I RH tachyzoites infecting HFF cells monolayer at the micromolar range ($IC_{50} = 1.79$ μM), albeit the first inhibitory effects can be observed at 0.5 μM concentration. This result was related mainly to the activity of the compound against TgGCN5-B that was isolated in order to evaluate its binding

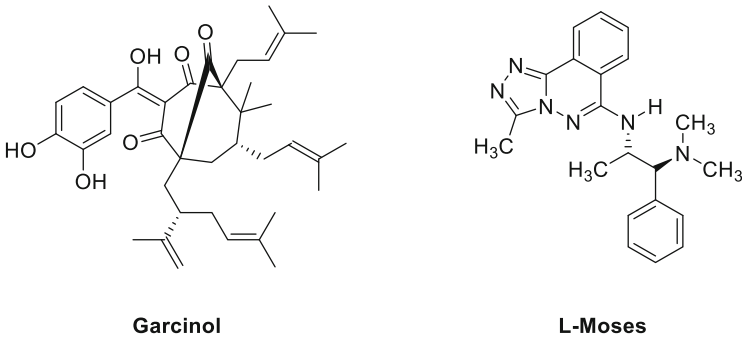


**Fig. 9** Structures of garcinol and L-Moses, inhibitors of TgGCN5

with the inhibitor. Interestingly, Western blot analysis performed after KAT assay (consisting in a mixture of KAT, acetyl-CoA, histone H3 and increasing concentration of the inhibitor) demonstrated the ability of garcinol to reduce H3 acetylation by TgGCN5-B. Similar outcomes were observed at the cellular levels because the tachyzoites exhibited reduced H3 acetylation, being this histone the preferred substrate of this KAT. Interestingly, also autoacetylation of TgGCN5-B showed to be reduced; albeit the effect of this modification is not clear, it could influence the activity of the enzyme representing a further control in gene expression. Garcinol led to a dysregulated expression of genes recognized as being under TgGCN5-B control. This effect, observed at the concentration of garcinol of 1 μM, was extended to a much larger array of genes at higher concentrations (2 μM), leading to catastrophic cascade due to aberrant gene expression. At the concentrations useful to kill *Toxoplasma,* impacts on human cells (HFF) were not detected accounting for selective toxicity of the compound. Unfortunately, in in vivo experiments (mice infected with Type I RH strain) garcinol at 10 mg/kg of body weight did not protect from acute infection the mice, albeit these results could be related to the pharmacokinetic properties of garcinol. In a successive work the same research group evaluated the opportunity to exploit the bromodomain of TgGCN5, the site designated at the binding of acetylated residues, as targetable element of the enzyme. Transgenic parasites of Type I RH strain were developed, at first. These cells expressed TgGCN5-B having mutations at the bromodomain and losing the ability to bind acetylated H4. In a standard plaque assay these mutations resulted in a 35% decreased lysis of the host cells, underlying the importance of the bromodomain for enzyme functionality. Further confirmations came from the use of a potent bromodomain chemical probe, L-Moses (Fig. 9), that inhibited the ability to bind acetylated H4 of TgGCN5-B at 1 μM concentration, while in in vitro assay performed against RH strain parasites (expressing a β-galactosidase [104]) showed inhibition of parasite proliferation with an $IC_{50} \sim 0.6$ μM. The dependence of these results by the bromodomain blocking has been confirmed by tests performed on cell line parasite constitutively expressing TgGNC5-B that conferred resistance to the drug.

## 6 Conclusions

Toxoplasmosis is the parasitic infection caused by the obligate intracellular parasite *T. gondii*. This pathogen possesses three different stages of life, namely: (1) sporozoites found in oocysts, (2) tachyzoites the replicative form, and (3) bradyzoites, the slow replicating form living in tissue cysts. Although the symptoms attributed to this infection are absent or quite mild, resembling flu illness, in immunocompetent patients it could be dangerous (people affected by HIV, patients who received a received haematopoietic stem cells or a solid organ transplant), leading to severe outcomes and/or death. To date, the clinical therapy of toxoplasmosis is still based on the use of drugs developed more than 50 years ago and endowed with high toxicity and ineffectiveness against bradyzoites, thus preventing the complete eradication of the parasite.

Albeit epigenetic drugs have been developed mainly to affect human cells regulatory mechanisms, their proposal or repurposing as effective treatment of toxoplasmosis should be considered. Indeed, these compounds are able to affect intimate mechanisms of gene expression control; so, their activity affect also the less responding form of the parasite, the bradyzoite one. One concern should be related to the selectivity of these drugs, because at this stage mostly of the evaluated compounds are molecules discovered for human proposal; so, their use could be associated with adverse events on the host cells. However, there are two important considerations that must be done. The first is that in most of the reported studies the concentrations required to affect parasite are lower than that used to obtain results on host cells; the second is related to the opportunity in the future to increase knowledge at structural level for epigenetic parasite targets in order to develop inhibitor tailored for these enzymes. In the light of the above, epigenetic treatments are still under inquiries, but the results seem to open a new route for toxoplasmosis treatment.

**Ethical Approval** This article does not contain any studies with human participants performed by any of the authors.

**Conflict of Interest** The authors declare that they have no conflict of interest.

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# Challenges and Promises for Obtaining New Antiprotozoal Drugs: What's Going Wrong?


**Alane Beatriz Vermelho, Mattia Mori, William A. Donald, and Claudiu T. Supuran**

## Contents



**Abstract** Infections by protozoa can cause some of the most serious human diseases, particularly in tropical regions. However, the number of available drugs used to treat such diseases tends to be limited with relatively high toxicity, and the vast majority of such drugs were developed in the 1920s to 1970s. The development of antiprotozoal drugs has been hindered owing in part to: (1) the highly complicated life cycles of such organisms and their ability to avoid innate immune defences; (2) challenges associated with culturing such organisms particularly in different phases of their growth and amplification; and (3) a lack of investment in biomedical research aimed at developing treatments for tropical diseases that do not tend to affect more affluent countries. Indeed, only three new drugs have entered into

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clinical trials in recent times, highlighting the tremendous gap in knowledge that should be bridged to more effectively treat protozoal infections.

**Keywords** Antiprotozoal drug, Drug resistance, Leishmania, Malaria, Parasitic protozoans

## 1 Introduction

Protozoans are microscopic, nonfilamentous protists widespread in most aquatic/soil habitats worldwide, being now considered as a paraphyletic group, with a complex evolutionary history [1, 2]. Most protozoans are heterotrophic organisms that acquire their nutrients from the environment, but there are examples of mixotrophic protozoans, such as *Paramecium* spp., which can also perform photosynthesis for their metabolic needs [1, 2]. Although such organisms are fascinating due to their multitude of phyla, genera, and species, many of which possess ecological and industrial importance, we will discuss here only the parasitic protozoans that infect humans and animals. They produce diseases which range from mild to moderate, such as those induced by *Toxoplasma gondii* or *Entamoeba histolytica*, to more serious conditions (e.g., infections due to *Cryptosporidium parvum*, *Giardia lamblia*, *Trichomonas vaginalis, Babesia* spp.) or very serious and widespread ones, such as malaria (infection due to at least five different species of *Plasmodium*), leishmaniasis (infectious protozoans are various species of *Leishmania*), Chagas disease (produced by *Trypanosoma cruzi*), and African sleeping disease (infection due to *Trypanosoma brucei*) [3–11], etc. Although rare, there are also several fatal protozoal diseases, such as those induced by amoebae belonging to the following three genera/species: *Naegleria fowleri* [12] *Acanthamoeba* spp. [13], or *Balamuthia mandrillaris* [13], for which few effective therapeutic approaches are available so far. Although the 12 protozoans genera which produce human disease are now well studied, there are still few available drugs for effectively treating these conditions. Furthermore, the drugs that are used have been available for decades, with high toxicity and low therapeutic indexes, and more concerning, extensive resistance to these treatment options has developed [3–5, 14–18]. Thus, what's going wrong? Why don't we have effective drugs for diseases that affect hundreds of millions of people worldwide, considering, for example, that for malaria alone there is an estimate of 229 million infections in 2019 linked to 409,000 fatalities [19]? We will attempt to answer these questions here.

## 2 Antiprotozoal Drugs in Clinical Use

One of the complications encountered when studying protozoans and approaches to inhibit their growth is related to the fact that most of them have quite complicated life cycles, with many different stages and also more than one host, with the vertebrate (human) being generally just one component in this intricate cycle [4–6, 16–18, 20, 21]. Taking as example again for malaria, which is provoked by at least five *Plasmodium* species which infect humans (i.e., *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae,* and *P. knowlesi*), the parasite is transmitted by mosquito bites (usually provoked by *Anopheles* mosquitoes) to vertebrates, infecting them, in a two-stage process. The first, pre-erythrocytic stage includes the following phases: (1) the plasmodial sporozoites are inoculated from mosquito salivary glands into the host through the bite; (2) the sporozoites reach the hepatocytes in the host's liver; (3) in the hepatocytes the parasite continues its development leading to schizonts which release into the bloodstream the merozoites, the final pathogenic form of the pre-erythrocytic stage. The second, asexual reproduction cycle (also known as blood-stage infection) includes: (1) the asexual multiplication of merozoites in red blood cells; (2) formation of immature ring stage trophozoites which consume the entire content of the invaded erythrocyte; (3) mature schizonts are formed in this way, which burst the red blood cell and release new merozoites into the bloodstream, which continue and enhance the infection of many other red blood cells. As it can easily be seen from this simplified description, *Plasmodia* have at least six different stages/phases during their life cycle, with the various forms of the pathogen present in different organs and tissues, but also with many different genes which are expressed in the different phases, and with a substantial capability to evade the host immune defences [4–6, 16–18, 20, 21]. This situation is also generally complicated for other pathogenic protozoans, which have complex life cycles and different secondary hosts (which can be different species of insects, for *T. cruzi, T. brucei,* and *Leishmania* spp.), or even other mammals, for example, *Toxoplasma gondii* (usually Felidae) [4, 5, 10]. In the case of *Cryptosporidium* spp., *Giardia lamblia*, *Entamoeba* spp., or *Trichomonas vaginalis* infections it seems that there is not an intermediate host, although these protozoans also have rather complex life cycles [6–9]. Overall, the complicated life cycles make it challenging to clearly identify promising druggable targets.

A second factor which is associated with difficulties in finding new/effective drugs for these pathogens is related to the fact that some of these organisms are difficult/impossible to grow in culture (e.g., *Cryptosporidium* spp.) [22] or their diverse stages/forms respond differently to drugs. Furthermore, some of these parasite stages are not at all prone to be grown in culture in order to allow a detailed study of the effectiveness of a drug during various stages of their life cycle [22–26].

Last but not least, many of the protozoan diseases are considered tropical diseases which affect a relatively low number of patients from poor countries. This is a very distorted reality, since as already mentioned, only malaria provokes a huge number of infections and many casualties each year. Furthermore, owing to climate change,

**Table 1** Diseases provoked by pathogenic protozoans and the therapeutic agents used for their treatment

| Disease | Pathogen | Drug |
|---|---|---|
| Malaria | *Plasmodium falciparum* | Quinine, chloroquine, primaquine; |
| | *P. vivax, P. ovale, P. malariae, P. knowlesi* | Artemisinin-based combination therapies |
| | | Atovaquone; proguanil; clindamycin; |
| | | Sulfadoxine, pyrimethamine, piperaquine |
| Leishmaniasis | *Leishmania* spp. | Antimonials (Sb(V) and Sb(III) derivatives) |
| | | Amphotericin B; pentamidine; paromomycin; |
| | | Miltefosine; antifungal azoles |
| Chagas disease | *Trypanosoma cruzi* | Nifurtimox; benznidazole |
| Sleeping sickness | *Trypanosoma brucei* | Pentamidine; suramin; nifurtimox; eflornithine |
| | | *Fexinidazole* |
| Trichomoniasis | *Trichomonas vaginalis* | Metronidazole; tinidazole |
| Giardiasis | *Giardia lamblia* | Metronidazole; tinidazole; furazolidone |
| Entamoebiasis | *Entamoeba histolytica* | Metronidazole; tinidazole; paromomycin |
| Toxoplasmosis | *Toxoplasma gondii* | Pyrimethamine; sulfadiazine; clindamycin |
| Cryptosporidiosis | *Cryptosporidium* spp. | *Nitazoxanide* |
| Babesiosis | *Babesia* spp. | Clindamycin; quinine; diminazene |

the enhance of temperatures in parts of Europe, North America, and Australia may soon create conditions for some of these "tropical" diseases to also appear (or reappear in some cases) in these parts of the world.

Table 1 shows the diseases provoked by protozoans in humans (and farm animals, in the case of *Babesia*) and the currently used drugs for their treatment. Only the two drugs shown in italics characters in Table 1, Fexinidazole and Nitazoxanide have been released for clinical use in the last 3 years [27, 28]. All other drugs shown in Table 1 were in fact discovered in the '30–'70s (except artemisinin and its derivatives, discovered in the '80s) and are characterized by rather high toxicity, low therapeutic index, and many side effects, although they are effective, especially in early phases of infection [4, 14–18]. Furthermore, just a limited number of chemotypes are present in the armamentarium of the antiprotozoal drugs, with the nitroazoles being predominant, followed by the dihydrofolate reductase and dihydropteroate synthase inhibitors [29] (Fig. 1). Although the recent approval of the two new agents Fexinidazole and Nitazoxanide is remarkable and demonstrates that relevant achievements can be obtained, both belong to the same class of nitroazoles.

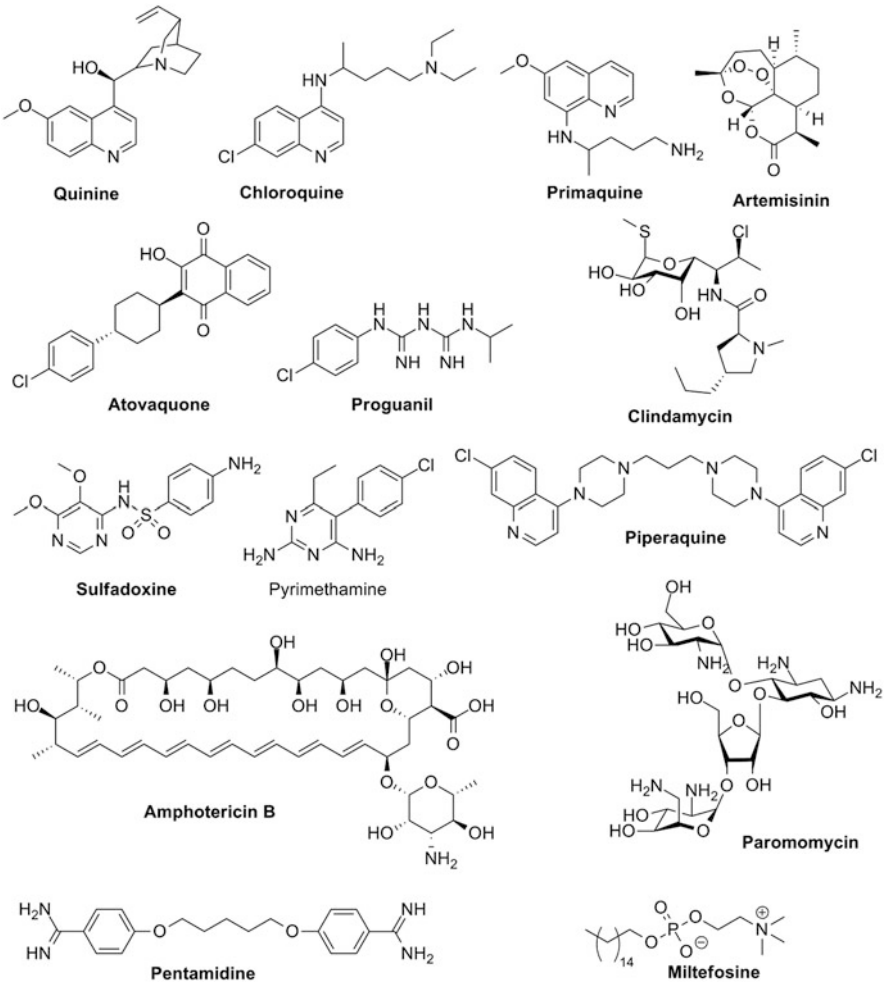



**Fig. 1** Chemical structure of drugs currently used in the therapy of human diseases caused by protozoans

## 3 New Drugs and Compounds in Clinical Trials

Acoziborole (SCYX-7158) (Fig. 2) is one of the few compounds in Phase III clinical trials for the treatment of *T. brucei* infection, being a benzoxaborole derivative, i.e., a totally new chemotype in the armamentarium of antiprotozoal drugs [30, 31]. Benzoxaboroles possess a range of pharmacological activities [32] in addition to antiprotozoal activity, including anti-bacterial, anti-fungal, antiviral as well as carbonic anhydrase inhibitory action [33–35].

Several new generation azoles such as ravuconazole and its prodrug (fos-ravuconazole) seem to be promising anti-*T. cruzi* agents [36], but there is

limited information regarding their clinical trials. It is rather disheartening to see that even for malaria, the worst of the documented protozoan diseases, most of the clinical trials that are registered in EU [37] deal with various vaccine candidates or combination therapies of existing drugs, but do not consider novel chemical entities.

## 4 Challenges for the Future after the COVID-19 Pandemic

The SARS-CoV-2 pandemic that emerged in late 2019 to early 2020 in China and spread all over the world [38, 39] should teach us that neglected diseases are a Sword of Damocles for the entire planet. The unprecedented (at least since 1918) crisis created by the outbreak of this viral disease demonstrated how susceptible the world is and how unprepared we were to tackle such a situation. As shown in this chapter, the number of protozoans is huge and many of them are poorly investigated and understood. Furthermore, such diseases are not restricted to tropical countries as some of the deadliest can be encountered in milder climates, including various *Amoeba* species that can provoke meningoencephalitis, which is difficult or

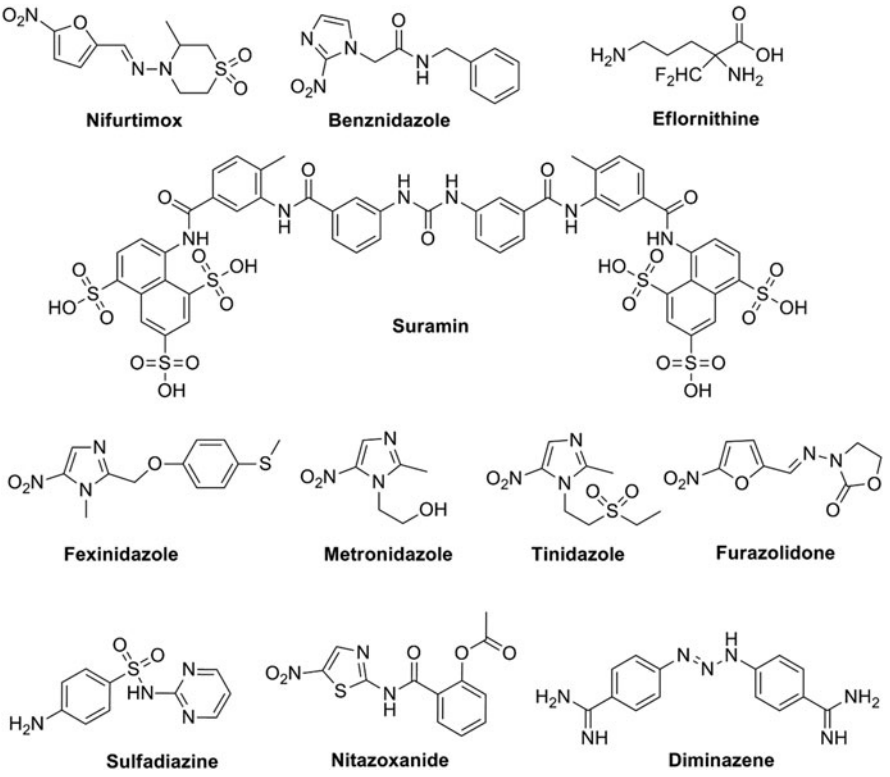
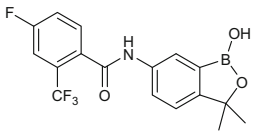



**Fig. 1** (continued)




Acoziborole

**Fig. 2** Chemical structure of Acoziborole

impossible to treat with currently available drugs [12, 13]. The number of available drugs is limited, restricted to a low number of clinical classes, and only a few stages of the parasite life cycle, which is by itself rather complex. The antiprotozoal drug targets are also quite limited, and although a relevant number of important discoveries have emerged by use of various 'omics methods over the previous two decades, there were essentially no significant translational studies from the lab to the clinic. Specifically, with the exception of two nitroazoles (see above), which were approved in the last 5 years, and the benzoxaborole derivative acoziborole (Fig. 2), no new drugs have emerged to treat protozoan-based diseases. What is going wrong? In addition to the challenges outlined above, there is also the perception that these are tropical diseases that will not affect people in affluent countries in which pharmaceutical research and large companies tend to be highly active. However, the tragic events of the last 18 months should remind us that this is no longer the case. Why do drug companies not invest in developing new antiprotozoal drugs, considering that the available ones are of low effectiveness and can have high toxicity? This situation should change, given the many interesting discoveries from academic researchers based all over the world that have emerged over recent decades, many of which are presented in the chapters of this book. By presenting an update of the state of the art in such diseases for nearly all protozoan infections, the current and broad gap in knowledge that needs to be bridged to develop excellent drugs for the treatment and management of these pathologies will become clearer.

**Compliance with Ethical Standards** *Conflict of Interest*: The authors declares that they have no conflict of interest.

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*Ethical Approval*: This chapter does not contain any studies with human participants or animals performed by the authors.

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# Correction to: Chagas Disease: Drug Development and Parasite Targets


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## Correction to: Chapter "Chagas Disease: Drug Development and Parasite Targets" in: Alane Beatriz Vermelho and Claudiu T. Supuran, Top Med Chem, https://doi.org/10.1007/7355_2021_143

The original version of the chapter was inadvertently published with errors in the names of these author names: **Giseli Capaci Rodrigues** and **Igor Almeida Rodrigues**. However, the author names have now been corrected.

The incorrect author names: Gisele Capaci Rodrigues and Igor de Almeida Rodrigues are now corrected as Giseli Capaci Rodrigues and Igor Almeida Rodrigues, respectively.

The updated online version of this chapter can be found at
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