Progresses in the Field of Drug Design to Combat Tropical Protozoan Parasitic Diseases

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Abstract: The progresses made in the field of drug design to combat tropical protozoan parasitic diseases, such as Chagas' disease, leishmaniasis, and sleeping sickness are discussed. This article is focused on different approaches based on unique aspects of parasites biochemistry and physiology, selecting the more promising molecular targets for drug design. In spite of the enormous amount of work on the above features, the chemotherapy for all of these diseases remains unsolved. It is based on old and fairly not specific drugs associated, in several cases, with long-term treatments and severe side effects. Drug resistance and different strains susceptibility are further drawbacks of the existing chemotherapy. In this review article, a thorough analysis of selected molecular targets, mainly those that are significantly different compared with the mammalian host or, even, are not present in mammals would be described in terms of their potencial usefulness for drug design. Therefore, this article covers rational approaches to the chemotherapeutic control of these parasitic infections, such as the progresses in the search for novel metabolic pathways in parasites that may be essential for parasites survival but with no counterpart in the host. Ergosterol biosynthesis is a very interesting example. There are many enzymes involved in this biosynthetic pathway such us squalene synthase, farnesylpyrophosphate synthase, and other enzymes that are able to deplete endogenous sterols will be treated in this article. The enzymes involved in trypanothione biosynthesis, glutathionyl spermidine synthetase and trypanothione synthetase do not have an equivalent in mammals, and therefore it can be predicted low toxicity for compounds that are able to produce highly selective inhibition. Trypanothione reductase (TR), glyceraldehyde-3-phosphate dehydrogenase, dihydrofolate reductase, prenyltransferases, ornithine decarboxylase, etc, will be thoroughly analyzed.

The design of specific inhibitors of such metabolic activities as possible means of controlling the parasites without damaging the hosts will be presented. The recent advances in the biochemistry of pathogenic parasites including the discovery of novel organelles will be discussed.

INTRODUCTION

Infections provoked by trypanosomatid and apicomplexan protozoa such as African and American trypanosomes, parasites from the *Leishmania* genus, and parasites causing malaria, toxoplasmosis and cryptosporidiosis are among the most prevalent parasitic diseases worldwide and are accountable for serious health and socioeconomic problems where all of these diseases are endemic.

The existing chemotherapy for these diseases like American trypanosomiasis (Chagas' disease), human African trypanosomiasis (sleeping sickness), leishmaniasis, malaria, toxoplasmosis, and cryptosporidiosis is not satisfactory in terms of its lack of effectiveness and also due to the toxicity associated to long-term treatments with empirically discovered drugs, in most cases. Drug resistance and different strain sensitivity to the available drugs is another drawback for the clinically accessible chemotherapy. The lack of financial motivations does not encourage pharmaceutical companies to carry out a rigorous research and developmental program bearing in mind that all of these illnesses are intimately related to poverty and unsuitable housing resources; therefore, efforts to develop new drugs should be achieved largely by academic and governmental institutions [1]. In fact, the drug discovery costs for the pharmaceutical industry in order to introduce new compounds into the market has risen dramatically from the last decades. Therefore, in the absence of vaccines, new chemotherapies are needed urgently to help in the prevention and control of these parasitic diseases. The main focus of this article is the use of unique features of the biochemistry and physiology of the different etiologic agents for the mentioned tropical diseases as well as in potential antiparasitic drugs that have been developed for other uses in humans, and, for that reason, have been proved to have few side effects and to be innocuous to humans.

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CAUSATIVE AGENTS

Trypanosoma cruzi

Chagas' disease is an endemic disease widespread from southern United States to southern Argentina. It has been estimated that around 18–20 million people are infected with the hemoflagellated protozoan *Trypanosoma cruzi*, the responsible agent of Chagas'disease [2]. The World Health Organization considers this serious illness as one of the

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major parasitic diseases [2]. In rural areas this disease is transmitted by reduviid bugs such as *Rhodnius prolixus* and *Triatoma infestans* as a consequence of their blood-sucking activity [3]. As other kinetoplastid parasites, *T. cruzi* has a complex life cycle possessing three main morphological forms. It multiplies within the insect gut as an epimastigote form and is spread as a non-dividing metacyclic trypomastigote from the insect excrements by contamination of intact mucosa or wounds produced by the blood-sucking activity of the vector. In the mammalian host, *T. cruzi* proliferates intracellularly in the amastigote form and is next released into the blood stream as a non-dividing highly infective trypomastigote that can either invade other tissues or can infect the respective Chagas' disease vectors closing the cycle [3,4]. Transmission of Chagas' disease could also occur *via* the placenta or by blood transfusion [5–7]. This latter mechanism is responsible for the occurrence of Chagas' disease in developed countries where the disease is not endemic. Actually, in the last few years, this illness has been encountered, in the United States, as a consequence of transfusion of contaminated blood from immigrants [5–7]. This disease has an acute phase, which may take place nearly unnoticed, although rarely it can lead to fatal meningoencephalitis or acute myocarditis, predominantly in children; an indeterminate asymptomatic phase, which can continue for more than ten years or even for the entire life of the infected individual; finally, a chronic phase, associated with heart problems or enlargement of hollow viscera (esophagus and colon) that may lead to death. As it was mentioned in the introduction, the chemotherapy for Chagas' disease at hand is still deficient [8,9]. It is based on two drugs empirically discovered, nifurtimox ((4-([5-nitrofurfurylidene]-amino)-3-methylthiomorpholine-1,1-dioxide, **1**), now discontinued, and benznidazole (*N*-benzyl-2-nitro-1-imidazoleacetamide, **2**). Although both of these compounds are able to cure at least 50% of recent infections as indicated by the disappearance of symptoms, and negativization of parasitemia and serology, they have important drawbacks. For example, (a) selective drug sensitivity on different *T. cruzi* strains; (b) these agents also produce serious side effects including vomiting, anorexia, peripheral neuropathy, allergic dermopathy, etc; long-term treatment is an additional disadvantage [10–12]. Moreover, these compounds are not effective in the chronic stage of the disease. In addition, there are a number of uncertainties concerning gentian violet (*N*-{4-bis[[4-(dimethylamino)-phenyl]methylene]-2,5-cyclohexadien-1-ylidene}*N*-methyl-methanaminium chloride, **3**), the only drug available to prevent blood transmission of Chagas' disease, because it is carcinogenic in animals [13,14]. This drug was empirically discovered for this purpose some decades ago [15] (Fig. **1**).

For the above reasons, it is required the development of new drugs that are more effective and safer than those currently available. There is a considerable amount of work in the search of unique aspects of the biochemistry and physiology of *T. cruzi.* These studies have led to the finding of specific molecular targets for drug design [16–21]. The rational chemotherapeutic approaches are focused on the metabolic differences between this pathogenic microorganism and mammals. Then, it can be anticipated that the selective inhibition of a biosynthetic pathway that leads to a crucial metabolite for parasite survival would not have any significant toxic effect for the host. Among them, sterol biosynthetic pathway, protein prenylation, thypanothione biosynthesis, trypanothione reductase, and other targets will be discussed in this article.

Trypanosoma brucei

African trypanosomes are among the most ancient eukaryotic organisms known [22,23]. Human African trypanosomiasis or sleeping sickness is caused by two kinetoplastid flagellates: *T. brucei rhodesiense* and *T. brucei gambiense*, which are subspecies of *T. brucei*. *T. brucei brucei* is generally considered exclusively an animal pathogen. *T. brucei* belongs to the order Kinetoplastida, family Trypanosomatidae, genus *Trypanosoma*, section salivaria and subgenus *Trypanozoon* [24]. The three members are phenotypically extremely alike, being morphologically identical and sharing major biochemical aspects. The number of new cases yearly is around 500,000 people, while 60 million individuals are at risk. Sleeping sickness is fatal if untreated and has re-emerged in recent years. Four drugs are clinically available for the treatment of this disease: melarsoprol (**4**), suramin (**5**), pentamidine (**6**), and eflornithine (**7**) [25]. The first three compounds were introduced more than half a century ago, while eflornithine was registered in 1990. However, this compound is only effective when sleeping sickness is caused by *T. b. gambiense*. Most of these compounds are toxic and lack efficacy. Parasite resistance is another important drawback for these drugs [25]. The corresponding chemical structures are illustrated in (Fig. **2**).

As other kinetoplastid parasites, *T. brucei* is characterized by the presence of a distinctive organelle named

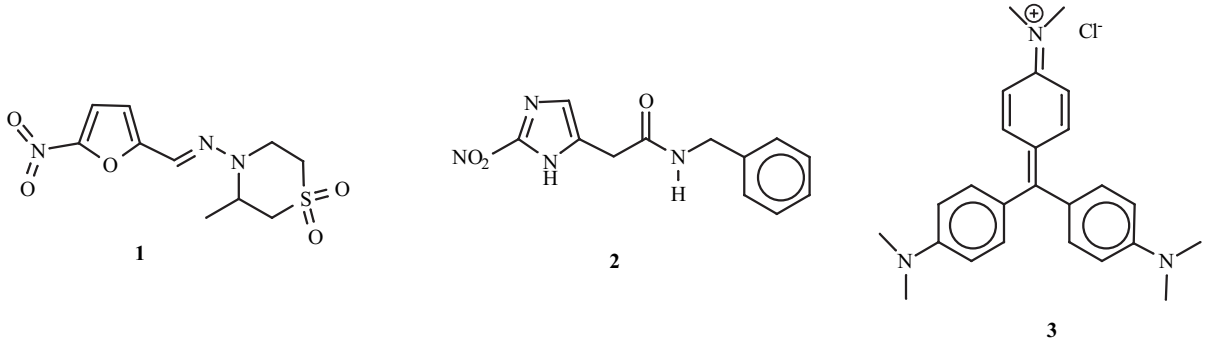


Fig. (1). Existing drugs used for Chagas' disease chemotherapy.

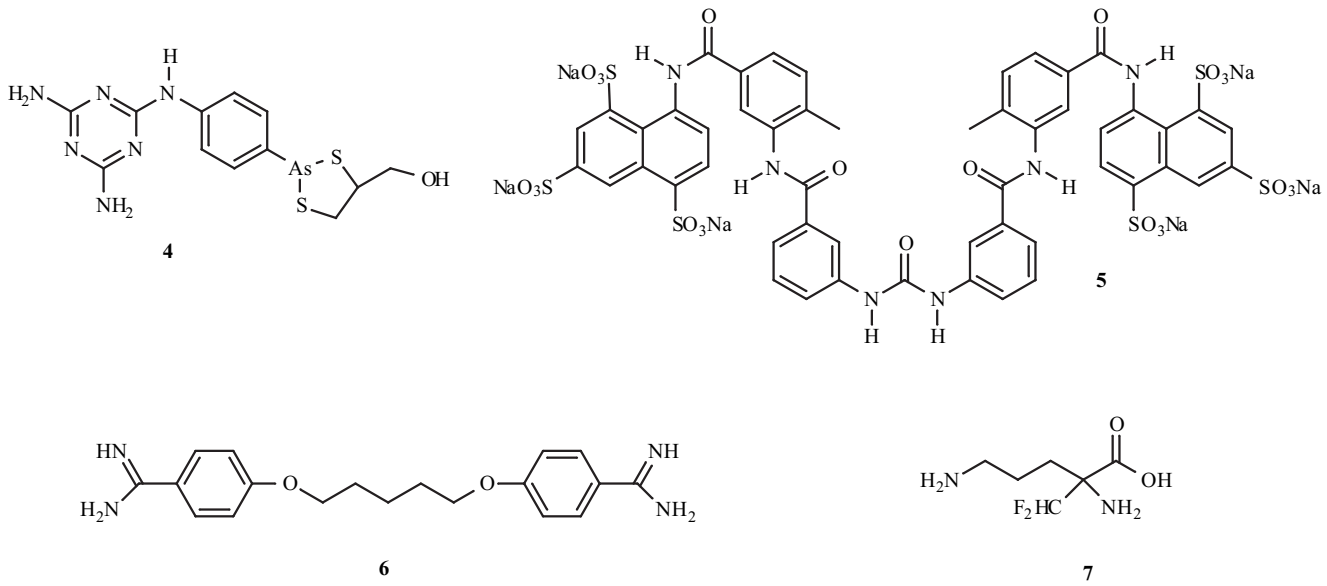


Fig. (2). Existing drugs used for sleeping sickness chemotherapy.

the kinetoplast. This DNA-containing organelle is located in the complex mitochondrion. The kinetoplast-mitochondrion complex differs both morphologically and functionally among the different forms of the parasite that exist at the different stages of the life cycle. Other organelles, including a Golgi apparatus, a nucleus with nucleolus and peripheral chromatin, an endoplasmic reticulum, glycosomes, acidocalcisomes, and a basal body and flagellar pocket, from which extends a single flagellum have been identified. The parasite also possesses a cell membrane, which is a complex grid of microfilaments and microtubules. Surrounding the cell membrane outer surface is covered with a single glycoprotein, the subject of antigenic variation [25].

Other important features of this parasite are the modest capability to synthesize aminoacids (acquired directly from the host); synthesize polyamines compounds, which are essential for proliferation and differentiation of the bloodstream stages, and lack glutathione reductase [26]. The main thiol compound is a conjugate called trypanothione [bis(glutathionyl)spermidine]. The trypanothione metabolism plays several key roles in trypanosomal survival.

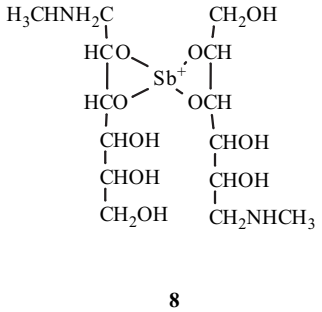
T. brucei is transmitted by several species of tsetse flies of the genus *Glossina*. Development of *T. brucei* in the insect initiates when an uninfected fly bites an infected vertebrate, ingesting *T. brucei* (trypomastigotes). The life cycle of *T. brucei* [27] involves cyclic transmission between a mammalian host and an insect vector. The vector takes a blood meal from mammals injecting metacyclic trypomastigotes, which transform into bloodstream trypomastigote that are transported to other sites. This form proliferates by binary fission in different body fluids such as blood, lymph, spinal fluid. Trypomastigotes in blood are taken by the vector and transform into procyclic trypomastigotes in insect midgut. These forms multiply by binary fission. Procyclic trypomastigotes leave the midgut and transform into epimastigotes, which proliferate in salivary gland. Finally, epimastigotes convert into metacyclic trypomastigotes closing the cycle. Within each of these distinct environments the parasite undergoes a series of differentiation events characterized by morphological restructuring, changes in surface coat and biochemical adaptation. Life cycle progression is intimately linked to cell cycle arrested forms pre-adapted for transmission between the different hosts. Upon transmission to a new host the cell cycle block is released and trypanosomes resume division. Although marked morphological differences exist in parasite life cycle, the procyclic cell acts as a prototype for the basic architecture of *T. brucei*.

At the beginning of the cell cycle trypanosomes possess a single flagellum, nucleus, kinetoplast and mitochondrion and must ensure the faithful duplication and segregation of all these organelles to produce two viable daughters [28]. Regulatory pathways controlling the eukaryotic cell cycle involve regulatory proteins such as cyclins, cyclin dependent kinases and cyclin dependent kinases inhibitors possessing distinct roles in the different life cycle stages [29–32].

Leishmania spp.

The leishmaniasis are a complex of clinical diseases produced by at least 17 species of the protozoan *Leishmania*. This parasite belongs to the order Kinetoplastida, family Trypanosomatidae [33]. The parasite exists in two morphological forms: in the mammalian host the parasite proliferate intracellularly as amastigotes within a phagolysosome compartment of macrophages, while extracellular flagellated promastigotes proliferates in the gut of their sand fly vectors [33–36]. The female sand fly probes with its proboscis from which it feeds. It becomes infected when eats blood containing amastigote-infected macrophages. Amastigotes convert to promastigotes in the gut of the sand fly at ambient temperatures. Promastigotes multiply and differentiate through a series of intermediate stages, including procyclic, nectomonad and haptomonad forms, before emerging as infectious metacyclic promastigotes [37]. There are minor ultrastructural differences in size and shape of promastigotes and metacyclic

promastigotes, which can not be differentiated on morphologic basis. The spectrum of disease has traditionally been divided into three major syndromes: cutaneous (CL), mucocutaneous (MCL) and visceral leishmaniasis (VL) [35,36]. *L. donovani* is the causative agent of visceral leishmaniasis, while *L. major*, *L. tropica*, *L. aethiopica*, *L. braziliensis*, *L. panamensis*, *L. amazonensis* and *L. mexicana* produce cutaneous leishmaniasis [36]. *L. chagasi* is the most important agent for visceral leishmaniasis in the New World [38–40]. Twelve million individuals suffer from human leishmaniasis worldwide with an incidence of 500,000 cases of VL and 1.0–1.5 million cases of CL per year and 350 million people at risk focused at tropical and sub-tropical regions [41,42]. Bearing in mind that primary cured infection leads to protection against further infection, the development of vaccines should be possible. Unfortunately, effective vaccines are not available up till now. Moreover, the incidence of leishmania/HIV co-infection has been increased worldwide due to the occurrence of visceral leishmaniasis in urban areas [43]. Leishmania parasites and HIV may also be transmitted as a consequence of sharing needles and syringes among intravenous-drug users [44]. Chemotherapy for leishmaniasis is still deficient. It is based on old drugs associated to long-term treatments, parasite resistance, and different drug sensitivity [45]. The primary drugs currently in use include pentavalent antimonials; these drugs require long-term treatments and possess the disadvantage of drug resistance [46]. Sodium stibogluconate (pentostam, 2,4:2',4'-*O*-(oxydistibylidyne)bis[D-gluconic acid] Sb,Sb'-dioxide trisodium salt nonahydrate) and meglumine antimoniate (glucantime; **8**) are pentavalent antimonials formulation currently being used for the treatment of leishmaniasis; although these drugs constitute the main antileishmanial chemotherapy, their precise molecular mechanism of action and the identity of the actual biologically active components are uncertain. Recent results would suggest that Sb compromises the thiol redox potential of the cell by inducing the efflux of intracellular thiols and by inhibiting trypanothione reductase [47]. In order to be active, it is well likely that Sb^{5+} has to be converted to Sb^{3+}; however, the site of this conversion is not known. A parasite-specific enzyme (TDR1) that catalyses the conversion of Sb^{5+} to Sb^{3+} using glutathione as a reducing agent has been recently reported [48]. In addition, a new enzyme (ACR2), which reduce Sb^{5+} was characterized in *Leishmania* and increase the sensitivity of *Leishmania* cells to Sb^{5+} [49]. The second line drugs, pentamidine (**6**), azole derivatives and amphotericin B (**9**), are very toxic and difficult to administer because of their long-term parenteral administration [50]. Parasite resistance is another drawback for pentamidine [51]. Although pentamidine cellular target is still unknown, parasite resistance has been associated to intracellular changes in arginine or polyamine transporter concentrations [52]. Recent studies have indicated that pentamidine gathers in the mitochondria enhancing the efficacy of mitochondrial respiratory chain complex II inhibitors [53–55]. Miltefosine (**10**), a former antitumor agent, is used at the present time in India for oral treatment of VL [56]. It is believed that the mode of action is associated with changes in alkyl-lipid metabolism, and phospholipids biosynthesis [56]. In the promastigote stage miltefosine could induce a cell death process-like apoptosis [57]. The major drawback of meltifosine is its teratogenicity, but parasite resistance has not yet been reported [58]. This compound seems to block sterol biosynthesis of *T. cruzi* either of the epimastigote or the amastigote forms of the parasite [59]. Two interesting drugs under clinical trials are the aminoglycoside



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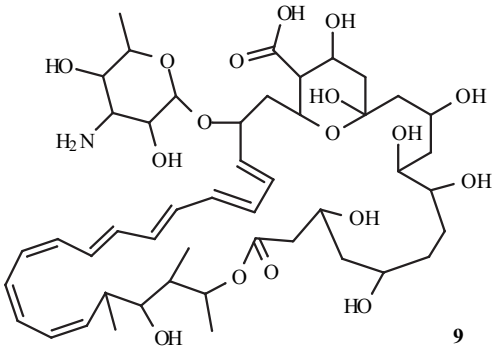

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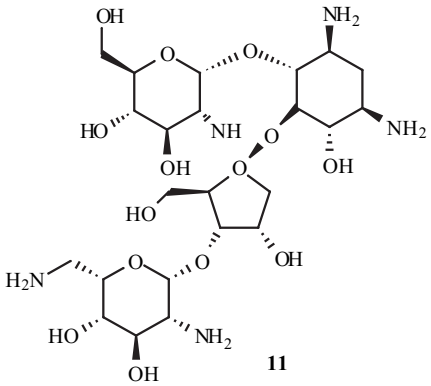

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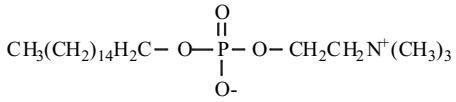

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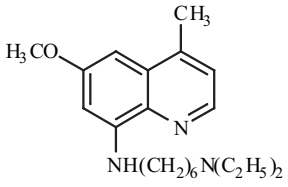

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Fig. (3). Existing drugs clinically employed for the treatment of leishmaniasis.

aminocyclitol antibiotic paromomycin (**11**), and the 8-aminoquinoline derivative sitamaquine (**12**). Paromomycin was identified as an antileishmanial drug in the 1960s and has *Leishmania* ribosomes as a potential primary target [60]. Variations in membrane fluidity and lipid metabolism are other effects observed [61]. The chemical structure of antileishmanial drugs is illustrated in (Fig. **3**).

Little is known regarding the mode of action or resistance mechanisms of sitamaquine. This compound would act as a prodrug and metabolic product would be responsible for the antiparasitic activity [35]. The above arguments justify investigations oriented to develop new antileishmanial agents.

Toxoplasma gondii

Toxoplasma gondii is a complex eukaryotic parasite that would be able to have all the required system for an independent life; however, *T. gondii* has adopted an essential intracellular survival. *T.gondii* causes a broad spectrum of disease but most infections are asymptomatic. The parasite actively penetrates host cells, sets up a privileged compartment in which it replicates and finally kills the cell. *T. gondii* is a member of the phylum Apicomplexa, class Sporozoa, subclass Coccidia, order Eucoccidia and suborder Eimeria [62].

It exists some evidences that *T. gondii* has plant-like characteristics. Some genetic elements may derive from a member of the green algae [63,64]. A membrane-bound, plastid-like structure, the apicoplast, contains a 35 kb circular genome and can be specifically inhibited by ciproflozacin, clindamycin and macrolide antibiotics. Many of the plastid genes have been transferred to the nucleus and may explain the plant-like character of *T. gondii* structural proteins such as tubulin. In addition, the parasite expresses enzymes of the shikimate pathway, essential for the synthesis of folate, ubiquinone and aromatic amino acids in algae and plants [65].

There are two asexual forms that can cause disease in humans. Tachyzoite form can invade all types of cells and divides rapidly leading to cell death. The bradyzoite form divides slowly and forms cysts in muscle and brain. The sexual cycle takes place in the superficial epithelium of the small intestine of members of the cat family. Oocysts, which are shed in feces of recently infected cats, remain in the upper soil horizon, where they may contaminate skin and may be ingested by hand-to-mouth transmission or on raw vegetables. Oocysts require for at least 12 hours in order to complete sporulation, afterwards they are infectious by mouth.

DRUG TARGETS AGAINST TRYPANOSOMIASES

Sterol Biosynthesis Inhibitors

Sterol biosynthesis in trypanosomatids proved to be an interesting target for the design of new drugs not only for fungi but also for different pathogenic parasites [66–68]. Sterol biosynthesis in parasites differs from that in mammalian hosts in that the final product is ergosterol rather than cholesterol, the main sterol present in the mammals [66–68]. Depletion of endogenous sterols such as ergosterol or 24-ethylcholesta-5,7,22-trien-3β-ol produces growth inhibition of the parasite; these metabolites can not be replaced by cholesterol; therefore, the selective inhibition of a crucial enzyme involved in the sterol biosynthesis of the parasite will impair *T. cruzi* proliferation. The blockage of this metabolic pathway in parasites has been extensively studied employing approved drugs for clinical use as broad-spectrum antifungal agents like imidazole and triazole derivatives. The ergosterol biosynthetic pathway of fungi is similar to that of pathogenic trypanosomes, and some of these antifungal drugs can be considered as eventual chemotherapeutic agents against American trypanosomiasis and leishmaniasis [69–73]. Moreover, it was found that *T. cruzi* as well as fungi and yeasts have rigorous requirements of precise endogenous sterols for cell viability and development. Certainly, some years ago, it was found that the most commonly used and effective antifungal agents were also potent inhibitors of pathogenic protozoa proliferation, for instance *Leishmania* spp and *T. cruzi* [74–77]. However, most of the clinically employed sterol biosynthesis inhibitors are not able to induce complete parasitological cure in human Chagas' disease and animal models [78,79]. Ketoconazole (**13**) [74] is a representative imidazole derivative that has a broad spectrum antifungal action. This compound has a dual action and is also a potent inhibitor of *T. cruzi* growth. The molecular target of this drug is the cytochrome P-450-dependent demethylation of lanosterol to form ergosterol, which is an essential metabolite as well as other 24-methyl sterols for cell proliferation as it was mentioned before [72,80,81]. Ketoconazole inhibits the enzymatic activity that catalyses the oxidative 14α-demethylation of lanosterol and other 14α-methyl sterols by binding to the protohaem Fe of citochrome P-450 14α-demethylase and by blocking a lipophilic site of the enzyme at the active site [82]. At concentrations of 1.0 μM ketoconazole completely blocks ergosterol biosynthesis in *T. cruzi* (epimastigotes) not affecting significantly growth rate of the parasite [76]. In addition, no ultra structural alterations are observed until endogenous ergosterol is depleted leading to drastic alterations of the kinetoplast-mitochondrion complex followed by mitochondria swelling and, finally, cellular lysis [83]. The intracellular forms of *T. cruzi* are even more susceptible to ketoconazole than the epimastigote forms; certainly, the concentration required to reduce the number of infective cells to a half and the minimum concentration to eradicate all the parasites are 0.1 nM and 10 nM, respectively [83]. This behavior can be reverted to some extent (cell growth inhibition, ultra structural alterations, etc.) by addition of exogenous ergosterol [83].

Terbinafine (**14**) is a different type of a broad-spectrum antifungal agent [84]. Similar to ketoconazole, this compound is also a potent inhibitor of *T. cruzi* proliferation [83,85] being effective against amastigotes at the very low micromolar level [81]. The target of this compounds is also the ergosterol biosynthetic pathway but at an early stage by inhibiting the enzymatic activity of squalene epoxidase (SE) [86]. Terbinafine is less potent than ketoconazole as an antiparasitic agent but has a strong synergistic effect, because

they act at different points of the ergosterol biosynthesis [81,83,84].

The bis-triazole derivative ICI 195,739 (**15**) [87] is another very attractive example about the ability of an antifungal agent to control *T. cruzi* proliferation [87]. This drug, as a racemic mixture, is two orders of magnitude more potent than ketoconazole against the epimastigote form of *T. cruzi*. However, its action is comparable to the latter compound against the intracellular form of the parasite being effective at the very low nanomolar level [82,85]. As addition of exogenous ergosterol does not reverse completely cellular lysis provoked by this bis triazole derivative, there is tantalizing evidence to believe that another mechanism of action is involved together with the blockage of sterol biosynthesis [85]. Further studies have indicated that the isomer responsible for the antifungal activity is the *R*-(+)-enantiomer [88–90]. In fact, the *R*–(+)-enantiomer, known as D0870, can induce radical parasitological cure of murine models of the acute and chronic stages of experimental Chagas' disease [69]. The mode of action of D0870 has been carefully studied as well [70]. Therefore, assays against epimastigotes indicate that the target of this drug is the sterol 14α-demethylase of *T. cruzi* as observed by accumulation of lanosterol, 4,14-dimethyl-ergosta-8,24(24^1)-dien-3-β-ol, and 24-methylene-dihydrolanosterol together with depletion of ergosterol and its precursors [70]. D0870 is able to eradicate completely the intracellular form of *T. cruzi* at a concentration of 10 nM [91]. This compound also blocks the sterol biosynthetic pathway as is observed by accumulation of lanosterol and 24-methyl-dihydrolanosterol. Moreover, a synergistic effect is observed when amastigotes are treated with D0870 and inhibitors of sterol $\Delta^{24(25)}$ methyl transferase such as 22,26-azasterol or 24(*R*,*S*),25-epiminolanosterol [92]. The *in vivo* efficacy of D0870 (not observed for ketoconazole) against Chagas' disease may be attributable to its remarkable pharmacokinetic properties [70]. In addition, this drug is also effective against a variety of *T. cruzi* nitroimidazole and nitrofurans resistant strains in *in vivo* assays [93].

Squalene synthase (SQS) is another interesting target to control diseases associated with infections caused by pathogenic parasites [94]. This enzyme catalyzes the first committed step in sterol biosynthesis by coupling two molecules of farnesylpyrophosphate to form squalene. The blockade of the sterol biosynthetic pathway at this step does not have an effect on other vital isoprenoids. On the other hand, the accumulated isoprenoids such as farnesyl pyrophosphate and precursors are readily metabolized with no toxic effects [95]. For the mentioned reasons, the human SQS have been studied as a cholesterol-lowering target [96], and its X-ray structure has been recently solved [97]. In addition, an effective and selective inhibitor of mammalian SQS such as 3-(biphenyl-4-yl)-3-hydroxyquinuclidine (3-biphenyl-4-yl-1-aza-bicyclo[2.2.2]octan-3-ol, **16**) [98] is able to impair both *T. cruzi* (epimastigotes) growth and *L. mexicana* (promastigotes) growth [94]. The control of these pathogenic parasites is associated with a non-competitive inhibition of the enzymatic activity of SQS at the low nanomolar range [94]. WC-9 (4-phenoxyphenoxyethyl thiocyanate, **17**) and other closely related compounds such as 2,4-dichlorophenoxyethyl thiocyanate (**18**) are potent and selective inhibitors against both the epimastigote form and the intracellular form of the parasite [99-104]. WC-9 arises as the main member of these aryloxyethyl derivatives being effective against amastigotes at the low nanomolar range and even more potent than nifurtimox and ketoconazole at the same assay conditions [99–101]. This compound is straightforwardly prepared from inexpensive 4-phenoxyphenol [99]. The molecular target of this compound was recently confirmed to be *T. cruzi* SQS [105]. In fact, the growth inhibitory effect of **17** is also associated to a depletion of *T. cruzi* endogenous sterols, ergosterol and its 24-ethyl analogue, and a simultaneous enhancement in the relative amount of cholesterol, taken from the growth medium [105]. At the minimum inhibitory concentration (MIC), defined as the minimal concentration required to inhibit growth by 99% after 96 hours, in this case, 1.0 μM, **17** produces a nearly complete disappearance of sterol contents with no accumulation of sterol intermediates or squalene [105]. These facts indicate a blockade of the ergosterol biosynthesis at the pre-squalene level [105]. Certainly, the effect of WC-9 on two key enzymes of the isoprenoid biosynthetic pathway such as *T. cruzi* farnesyl pyrophosphate synthase (*Tc*FPPS) [106] and *Tc*SQS, confirms this hypothesis. In fact, WC-9 is a potent inhibitor of both glycosomal and mitochondrial *T. cruzi* SQS with IC_{50} values of 88 and 129 nM, respectively, while it is basically devoid of activity against *Tc*FPPS (13% of inhibition at 40 μM) [101]. In conclusion, the aryloxyethyl thiocyanate motif has been identified as a pharmacophore that led to the development of many drugs. Compounds **17** and **18** arise as principal members of aryloxyethyl thiocyanate derivatives. Aryloxyethyl thiocyanates are not only lead drugs but also potential chemotherapeutic agents [99,100]. The 4-phenoxyphenoxy moiety has been found in other structurally related drugs as it is the case of matrix metalloprotease inhibitors [107], and also in a number of other drugs [108]. In addition and surprisingly, it has been found that juvenile hormone mimics that act against insects other than Chagas' disease vectors such as *Tenebrio molitor*, *Galleria mellonella*, *Dysdercus cingulatos*, and *Pyrrhocoris apterus* are moderate inhibitors of *T. cruzi* growth [109]. This dual action, that is, drugs having juvenile hormone activity and possessing cellular activity has been formerly observed exclusively in Chagas' disease vectors like *T. infestans* and *R. prolixus* together with *T. cruzi* cells [110]. Juvenile hormones are isoprenoid derivatives that are crucial for maintaining larval stages in insects and are responsible for the maturation of the reproductive system in the female [111].

Posaconazole (**19**) is a new experimental triazole derivative which present a broad-spectrum antifungal activity [112] targeting 24α-demethylase [71,113]. Posaconazole is an extremely potent inhibitor of *T. cruzi* proliferation; its biological action is associated with an exceptionally effective blockade of the sterol biosynthetic pathway [71]. Certainly, **19** is able to eradicate the intracellular amastigote form of *T. cruzi* from the host cells (Vero cells) at a concentration of almost two orders of magnitude lower than that required for ketoconazole [71]. Posaconazole is able to produce both animals survival together with blood sterilization more efficiently than **(+)–15** and significantly better than **13** in a murine model of acute Chagas' disease [71]. This drug also has trypanocidal activity in a variety of *T. cruzi* strains, still

in some benznidazole-, nifurtimox- and ketoconazole-resistant strains [113]. Such effectiveness is observed in murine models of the acute and chronic stage of American Trypanosomiasis [113].

Ravuconazole (**20**) is another potent and broad-spectrum antifungal agent, which also exhibits potent inhibitory action on *T. cruzi* proliferation. This drug blocks the sterol biosynthesis at the level of cytochrome P-450-dependent sterol C14α demethylase [114]. This compound acts at the low nanomolar level against the intracellular form of the parasite with vanishing effect in the development of the host cells indicating excellent selectivity for parasite cells [114].

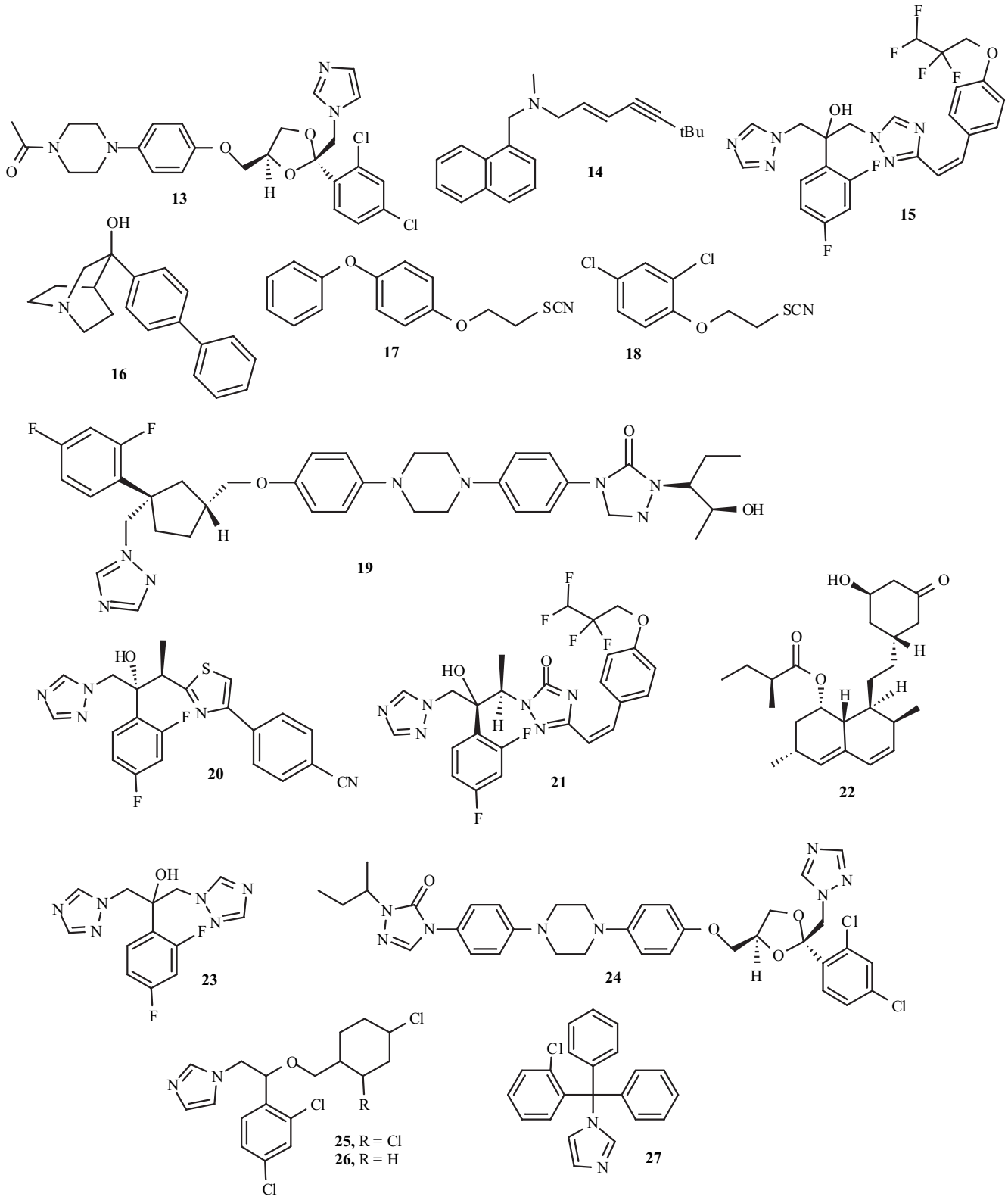
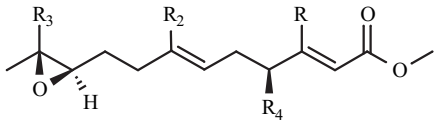


Fig. (4). Chemical structures of representative sterol biosynthesis inhibitors (SBI).

In spite of presenting such a powerful inhibitory potency *in vitro*, this compound is not satisfactory in *in vivo* assays due to adverse pharmacokinetics properties. Compound **21** also known as TAK-187 is another example of a triazole derivative possessing dual action as an antifungal and as a trypanocidal agent [115]. The mode of action of this triazole derivative is *T. cruzi* cytochrome P-450 dependent sterol C14α demethylase [115]. This drug is also very effective against amastigotes at the low nanomolar range and it has no effect on the viability and growth of the host cells [115].

3-Hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase), the first committed step in the isoprenoid biosynthesis, is an interesting target for drug design. Lovastatin (**22**) [116] is an approved cholesterol-lowering agent for humans and is a highly potent and competitive inhibitor of HMG-CoA reductase [117]. This compound exhibits antiproliferative action against *T. cruzi* (epimastigotes) in a dose-dependent manner [118]. In addition, lovastatin possesses a synergistic effect when used together with ketoconazole producing total elimination of the amastigote form of *T. cruzi* in Vero cells. Interestingly, lovastatin has no effect against intracellular amastigotes when used alone and at concentrations higher than 1.0 μM presents a deleterious effect to the host cells. A marked synergistic action of lovastatin and ketoconazole has also been observed in *in vivo* assays. HMG-CoA reductase is a soluble enzyme located in the glycosomes [119].

There are many other examples of antifungal agents that also display antiparasitic activity but to a lesser extent than those previously described. For example, fluconazole (**23**), itraconazole (**24**), miconazole (**25**), econazole (**26**), and clotrimazole (**27**) are additional ergosterol biosynthesis inhibitors and exert antiproliferative action against *T. cruzi* growth [72,82,120,121]. Clotrimazole has been considered as an antiproliferative agent for *Leishmania* spp. since many years ago [121]. Synergism of lovastatin has also been observed when used in combination with miconazole (*vide infra*) in biological experiments against *Leishmania* spp. [122]. The respective structures of the above sterol biosynthesis inhibitors of *T. cruzi* are illustrated in (Fig **4**), while the chemical structures of naturally occurring juvenile hormone of insects is presented in (Fig. **5**).



28, JH 0	$R_1 = R_2 = R_3 = Et$		$R_4 = H$
29, JH I	$R_1 = Me$	$R_2 = R_3 = Et$	$R_4 = H$
30, JH II	$R_1 = R_2 = Me$	$R_3 = Et$	$R_4 = H$
31, JH III	$R_1 = R_2 = R_3 = Me$		$R_4 = H$
32, 4-Me JH I	$R_1 = Me$	$R_2 = R_3 = Et$	$R_4 = Me$

Fig. (5). Naturally occurring juvenile hormone of insects.

Protein Prenylation

Protein prenylation arises as a specially attractive target for drug design in many tropical parasitic diseases [123–125]. Of special interest are bisphosphonic acid derivatives (compounds of general formula **33**) that are compounds structurally related to inorganic pyrophosphate in which a methylene group has replaced the oxygen bridge between the phosphorous atoms. Unlike pyrophosphate, geminal phosphonates present greater metabolic stability because they are not hydrolyzed by pyrophosphatases and are also stable to hydrolysis under acidic conditions. Some bisphosphonate derivatives are effective inhibitors of bone resorption and are currently being used for the treatment of several bone disorders such us osteoporosis, Paget's disease, complications associated with bone metastases and multiple myeloma, hipercalcemia provoked by malignancy, bone inflammation associated with rheumatoid arthritis or periodontal disease [126–131]. Representative bisphosphonic acid derivatives commonly employed for the treatment of bone disorders are illustrated in (Fig. **6**).

Bisphosphonates were formerly designed to mimic the chemical structure of pyrophosphate. In spite of having been used for more than thirty years, the target of these drugs, the isoprenoid pathway, has been recently elucidated [131–133]. Early work had postulated that these drugs were putative inhibitors of pyrophosphate-related metabolic pathways like protein prenylation, which occurs in pathogenic trypanosomes [134]. Protein prenylation is responsible for the attachment of farnesyl and geranylgeranyl units to the *C*-terminal cysteine residues of several proteins, such as the small GTPases such as Ras, Rac, Rab, and Rho, giving rise to farnesylated and geranylgeranylated proteins. These proteins are important signaling molecules involved in key cell processes for osteoclasts function [135]. The attached prenyl groups play an significant role in anchoring proteins to membranes and also take action in protein-protein interactions. Three enzymes have been identified in eukaryotic cells: protein farnesyl transferase (PFT) and protein geranylgeranyl transferases I (PGGT-I) and II (PGGT-II) [136]. Selective inhibition of PFT slows down human tumors growth due to farnesylation inhibition of oncogenic Ras [137]. This result led to the development of many FPT inhibitors [137] as potential antitumor agents. Interestingly, a dual action of these type of drugs is observed due to FPT inhibitors are also effective growth inhibitors against *T. cruzi* and *T. brucei* proliferation [130]. The molecular target of nitrogen-containing bisphosphonates in osteoclasts [138–140], plants [141], and *Dictyostelium discoideum* [142] is farnesyl pyrophosphate synthase (FPPS). This enzyme catalyzes the formation of the substrate for protein prenylation [143]. Inhibition of the FPPS enzymatic activity results in decreased content of sterols, dolichols and ubiquinones together with the inhibition of protein prenylation. It had been postulated that nitrogen-containing bisphosphonates, particularly, those nitrogen-containing bisphosphonates with the nitrogen atom at the C-3 position, would act as carbocation transition state analogues of isoprenoid diphosphates for isoprenoid biosynthesis [106,144]. However, some questions have been raised about this assumption because several nitrogen-free bisphosphonates, namely 1-alkyl-1,1-bisphosphonates also target FPPS [145–147].

The information that some representative FDA-approved bisphosphonates such as pamidronate (**37**) and alendronate (**38**) inhibited *T. cruzi* proliferation *in vitro* and *in vivo* without toxicity to the host cells [148] encouraged the search for innovative bisphosphonates to be used as antiparasitic

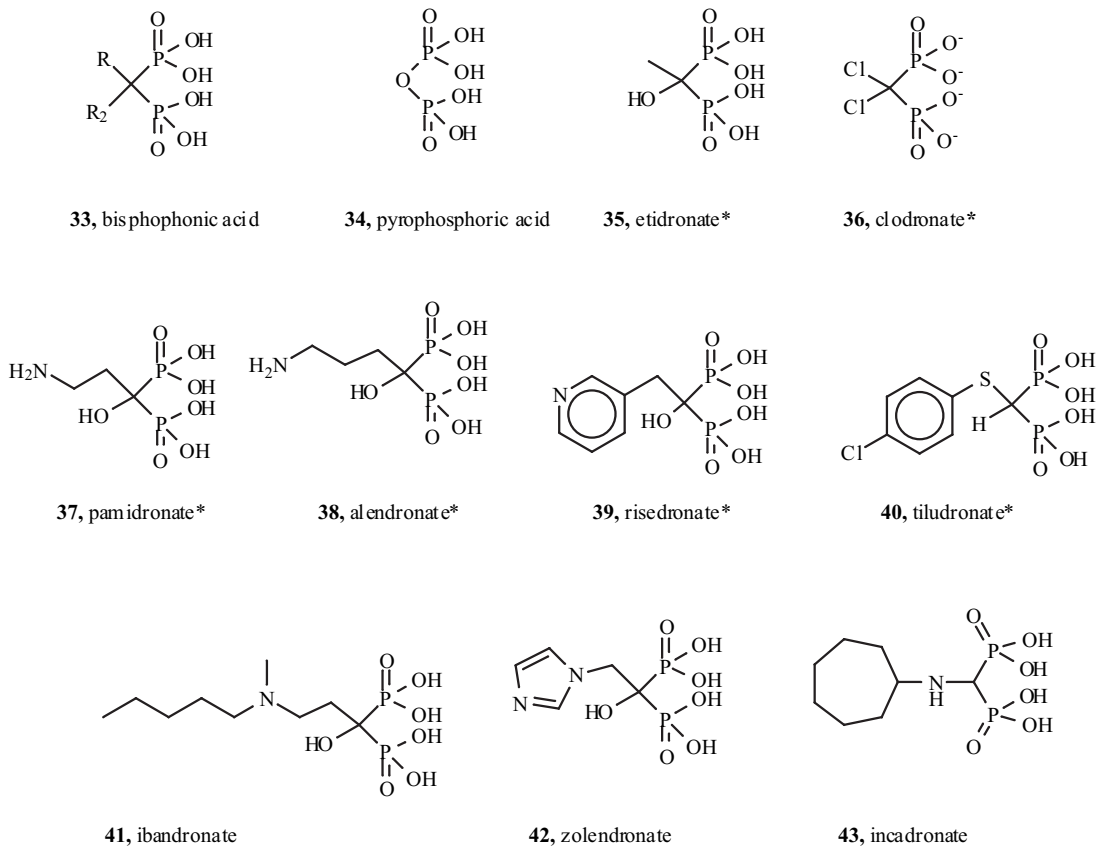


Fig. (6). Chemical structures of representative member of bisphosphonates currently employed for the treatment of bone disorders.

agents. In this context, it is possible to ensure that a number of bisphosphonates are potent growth inhibitors of several pathogenic trypanosomatids (*T. cruzi*, *T. brucei rhodesiense*, and *Leishmania donovani*) and apicomplexan parasites (*Toxoplasma gondii* and *Plasmodium falciparum*) [149,150]. Risedronate possesses IC_{50} values of 0.22 μM for *T. brucei rhodesiense*, and 0.49 μM for *T. gondii* tachyzoites [150]. In addition, bisphosphonates derived from fatty acids, which have no nitrogen atom present in their chemical structure, are potent inhibitors of *T. cruzi* proliferation at the same level than WC-9 [145,146]. For example, bisphosphonate derived from heptanoic acid (compound **44**) is a potent inhibitor against *T. cruzi* (amastigotes) proliferation [145], **44** is very efficient agaisnt *T. gondii* [147], and **45** is very efective against *P. falciparum* (IC_{50} = 0.83 μM) [151] (Fig. **7**). Taking into account that bisphosphonates derivatives are FDA-approved drugs for long-term treatment of bone disorders; it might be anticipated low toxicity for new compounds bearing the bisphosphonate moiety. Bearing in mind that the pharmacophore corresponded to the *gem*-phosphonate unit, and on the basis of the potent inhibitory action exhibited by many bisphosphonates, it can be anticipated that bisphosphonates are excellent drug candidates to be used as chemotherapeutic agents against a number of parasitic diseases.

Trypanothione Pathway

Prospects in antileishmanial and antitrypanosomal drug research have changed substantially since the discovery of trypanothione (**46**, $T(SH)_2$) [152]. The uniqueness of this metabolite and its biosynthetic pathway in parasites of the order Kinetoplastida, confer to the involved enzymes of its biosynthesis a great usefulness as a molecular target. Because of the absence of this metabolite in the host mammalian cells, there is an opportunity to design highly selective antiparasitic drugs against leishmaniasis and trypanosomiasis without toxic effects. Current chemotherapy is based on empirically discovered drugs that interact with parasitic metabolic pathways that have their corresponding counterparts in mammals; therefore, the efficacy of these drugs is sustained by the ability of the host to balance metabolic deficiencies with alternate pathways. In addition, trypanothione is essential for parasite survival for two

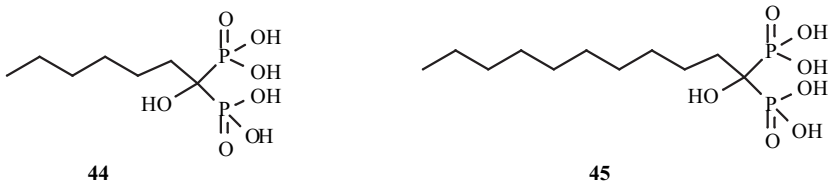


Fig. (7). Chemical structure of bisphosphonates derived from fatty acids.

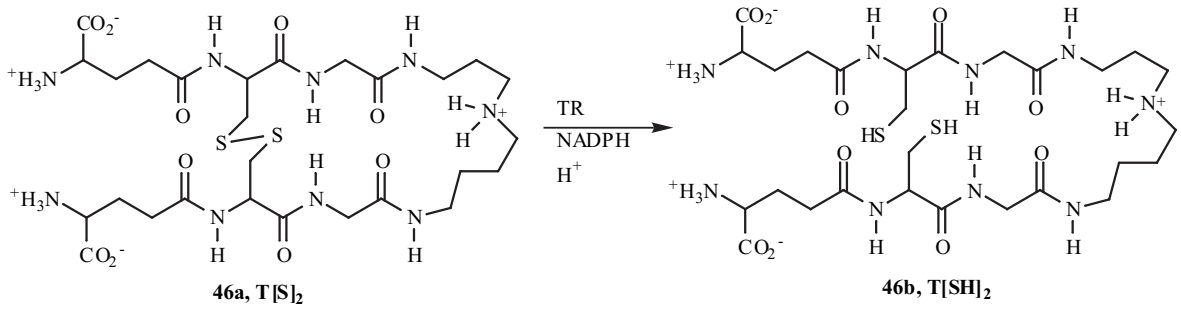


Fig. (8). Oxidized and reduced form of trypanothione (compounds **46a** and **46b**, respectively).

reasons: (a) its crucial role in regulating the cellular redox equilibrium, (b) pathogenic trypanosomes do not have an alternate mechanism to protect against the oxidative stress [153].

Chemical Structure and Physical Properties

Trypanothione is the bisconjugated product between the tripeptide glutathione (**47**) and the poliamine spermidine: N^1,N^8-bis-(L-γ-glutamyl-L-hemicystinyl-glycyl)spermidine. The cysteine residues present in the trypanothione moiety are involved in the interconversion between its oxidized form [**46a**, $T(S)_2$] and reduced form [**46b**, $T(SH)_2$] by cleaving and restoring an intramolecular disulfide bridge. Both structures have a +1 net charge at the physiologic p*H* (Fig. **8**) [17].

Redox potential of the $T(SH)_2/T(S)_2$ couple has been measured and compared to the glutathione/oxidized glutathione couple. There is only a slight difference: $E_0' = -0.242$ V for the $T(SH)_2/T(S)_2$ couple and $E_0' = -0.230$ V for the latter couple. In addition, association constants with trivalent organic arsenicals have also been measured and shown that trypanothione forms stable complexes with these well-known trypanocidal drugs [154]. Both the reduced and the oxidized forms of trypanothione seem to be flexible molecules in aqueous solution according to NMR conformational studies on a synthetic sample of this metabolite [155].

Metabolic Functions

Redox Cellular Equilibrium

Trypanothione has several functions in parasite metabolism such as the cellular redox equilibrium, the antioxidant defense, and the mechanism of defense against xenobiotics. All living aerobic organisms need to maintain their cellular redox equilibrium. In order to regulate this balance, some low molecular weight thiol, always present within these organisms, switch between the oxidized and reduced forms of these sulfur-containing species. In most cases, glutathione (GSH) is the thiol derivative involved in

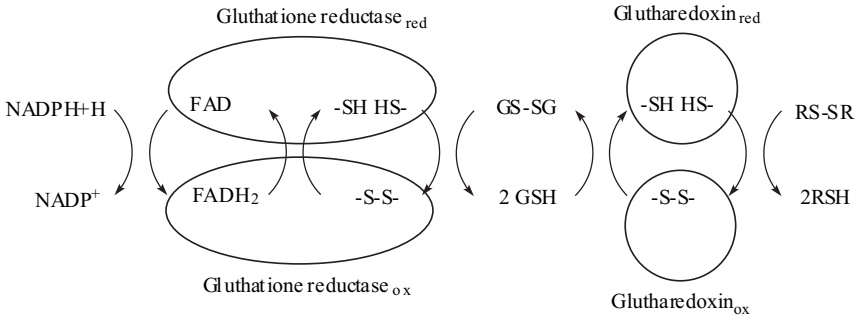


Fig. (9). Gluthatione reductase, gluthatione, glutharedoxin system.

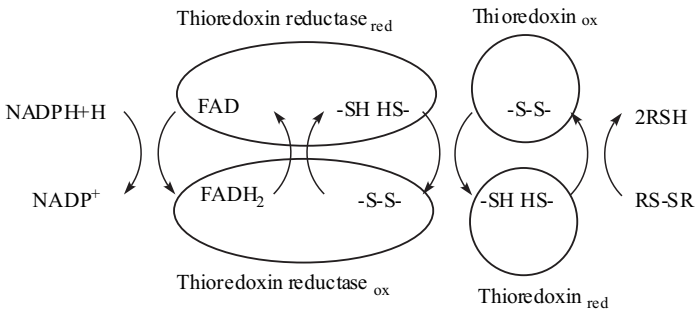


Fig. (10). Thioredoxin reductase, thioredoxin system.

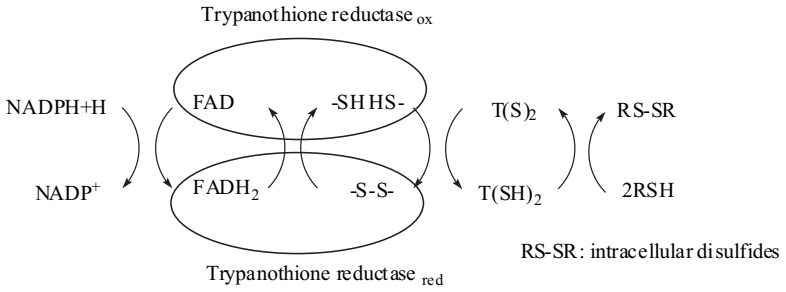


Fig. (11). Redox equilibrium system.

this process. {Prot $_{ox}$ + 2 GSH $\rightleftarrows$ GS-SG + Prot $_{red}$}. Therefore, the most important function of GSH is to avoid disulfide formation in intracellular proteins, because this effect could seriously alter their activity. Mammalian cells have two general mechanisms for disulfide reduction: *glutathione reductase, glutathione, glutharedoxin* (Fig. **9**) system and *thioredoxin reductase, thioredoxin* system (Fig. **10**).

Glutathione is also present in trypanosomatids. Nevertheless, it was observed that the NADPH dependent reduction of GSSG, GSSG-CoA and cysteine in crude cell extracts required the presence of a sulfur-containing cofactor of low molecular weight [156]. This cofactor, originally isolated from the non pathogenic trypanosomatid *Critidia fasciculata*, is characterized as N^1, N^8-bis(glutathionyl)spermidine (trypanothione). Furthermore, no glutathione reductase activity is found within the cells. It can be concluded that glutathione reduction takes place by a non-enzymatic disulfide interchange reaction between reduced trypanothione and oxidized glutathione. The enzyme responsible for the reduction of trypanothione was isolated and named trypanothione reductase [157]. This enzyme, which is highly specific for trypanothione, does not recognize glutathione as a substrate. The principal redox equilibrium maintenance system is illustrated in (Fig. **11**).

Antioxidant Defense

Trypanosomatids, as other aerobic organisms, are exposed to highly reactive oxygen species such as O_2^-, H_2O_2 and HO·. These substances are produced internally by cofactors like reduced flavines, quinones or thiols, and externally by the host immune system. HO· and RO· radicals can cause lethal damage if irreversibly react with DNA or cellular membrane lipids [158]. The only defense possible against radicals is to trap them by low molecular weight molecules such as vitamins A, C and E, uric acid, billirrubin or thiols, in particular glutathione or trypanothione; elimination of radical species is the last resource. In order to avoid this problem, the defense system maintains low levels of H_2O_2 and O_2^-. Superoxide dismutases (SOD) have been identified in *T. cruzi* [159], *L. donovani* [160], and *C. fasciculata* [154]. Overexpression of Fe-SOD in *T. cruzi* yields parasites more susceptible to redox drugs due to their lack of capability to detoxify H_2O_2 generated from O_2^- [160]. In addition, catalase and glutathione peroxidase are not present in parasites. Glutathione reductase activity is not found in trypanosomes, but a highly efficient trypanothione reductase activity is present instead [158]. All these findings are in agreement with the assumption that glutathione is a precursor of trypanothione, and the trypanothione-dependent hydroperoxide metabolism is the counterpart of the glutathione-dependent metabolism present in mammals. There are some differences between trypanothione and glutathione defense mechanisms, the redox cascades are different in both metabolisms, the $T(SH)_2$ reduction equivalent acceptor is a protein related to thioredoxin family, namely tryparedoxin, which acts as substrate for tryparedoxin peroxidase in trypanosomatids [153]. In other words, there are three distinct enzymes involved in the redox cascade of trypanosomatids, while only two take part in the mammalian redox cascade.

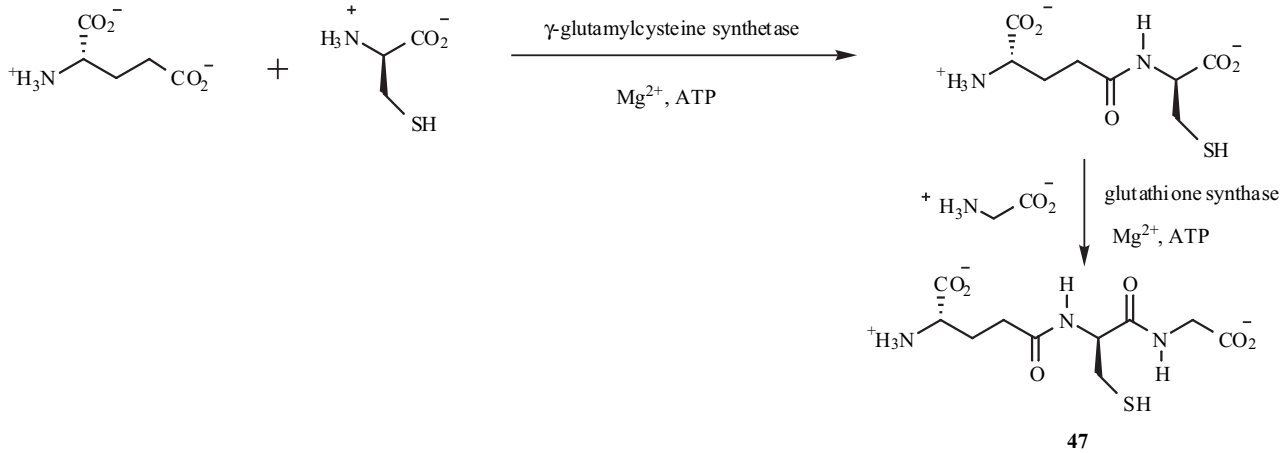


Fig. (12). Glutathione biosynthesis.

Defense Against Xenobiotics

In general, xenobiotics are hydrophobic substances of diverse chemical structures that are converted to less toxic and soluble compounds to be more easily excreted when conjugated with glutathione. This reaction is catalyzed by glutathione *S*-transferases, enzymes found in more developed organisms, while typanothione *S*-transferase activity is present in several parasites such as *C. fasciculata*, *L. major, L. infantum, L. tarentolae,* and *T. brucei*, associated to the 1B eukaryotic elongation factor (eEF1B) [161]. The eEF1B has been expressed, cloned and purified in *L. major* [162] catalyzing conjugation between a variety of electrophiles with trypanothione and glutathionylspermidine, but not with glutathione. Once again, this finding suggests that trypanothione plays the same role in parasites that glutathione does in mammals.

Glutathione Biosynthesis

Glutathione biosynthesis is catalyzed by two ligases that use ATP as activation factor: γ-glutamylcysteine synthetase and glutathione synthetase. Both enzymes require Mg^{2+} as cofactor. Substrate specificity and kinetic properties have been studied for recombinant γ-glutamylcysteine synthetase in *T. brucei* and mammalian enzyme. Some differences are existent in the binding pocket [163], but there are no selective inhibitors available [164] (Fig. **12**).

Spermidine Biosynthesis

Spermidine (Spd, **48**) is biosynthesized from ornithine and methionine. Ornithine decarboxilase (ODC), *S*-adenosilmethionine synthetase, *S*-adenosilmethionine decarboxilase and spermidine synthetase are involved in this process as illustrated in (Fig. **13**).

Polyamines are essential for cell proliferation processes, differentiation and membrane functions. Hence, if their biosynthesis is inhibited, impair cell growth takes place. It has been recently shown that the polyamine spermidine is essential for normal proliferation of *C. fasciculata* [165]. However, it is still not clear for all kinetoplastid protozoa, if polyamines levels are due to parasite biosynthesis or to transport processes taken from external media. For example, Kierszenbaum *et al*. reported that ODC inhibitors diminished *T. cruzi* ability to infect mammalian cells [166]. Ariyanayagam and Fairlamb did not detect significant activities neither of ODC nor of arginine decarboxilase (ADC) in this parasite, concluding that *T. cruzi* takes polyamines form external media [167]. On the other hand, Hernández *et al.* reported significant ADC activity levels in crude cell extracts of *T.cruzi* [168]. Furthermore, it seems to be clear for *Leishmania* that the parasite is able to proceed with polyamines biosyntheses from ornitine [169]. Nevertheless, two specific carriers have been identified for spermidine and putrescine in *L. mexicana* and *L. donovani* (promastigotes and amastigotes) [170]. For the above reasons, it is clear that in the presence of transport processes, polyamine metabolism does not represent a suitable target for chemotherapeutic drug development.

Trypanothione Biosynthesis

The last two steps of trypanothione biosynthesis are the most relevant ones in terms of molecular targets, because they involve enzymes that have no counterpart in mammals. These flavoenzymes are ATP-dependent C:N ligases, which require Mg^{2+} as cofactor, catalyze the conjugation of spermidine with two molecules of glutathione to yield trypanothione. Since their discovery there have been some uncertainty about their identity and function. Even at the present time it is not yet completely clear how this conjugation takes place in all parasites of the order Kinetoplastida. Two possibilities are being investigated: one comprises two distinct enzymes (GspS and TryS) that catalyze sequentially the conjugation of GSH with Spd and the resulting product, glutathionylspermidine (**49**), with another molecule of GSH, and the other one involves only one enzyme (TryS), which is able to catalyze the synthesis of $T(SH)_2$ directly from GSH and Spd. (Fig. **14**)

Glutathionylspermidine Synthase and Trypanothione Synthase

First activity assays and purification of these enzymes were carried out on the non pathogenic parasite *C. fasciculata* [171]. Smith *et al.* adding one additional putification step isolated two distinct enzymes [172]. One

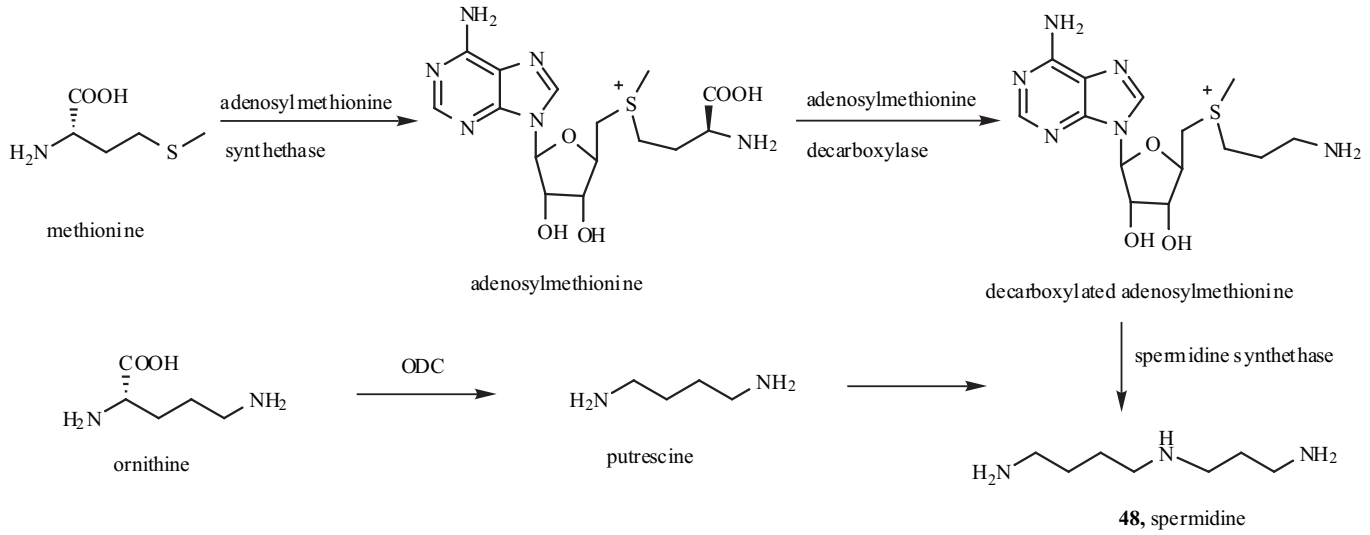


Fig. (13). Spermidine biosynthesis.

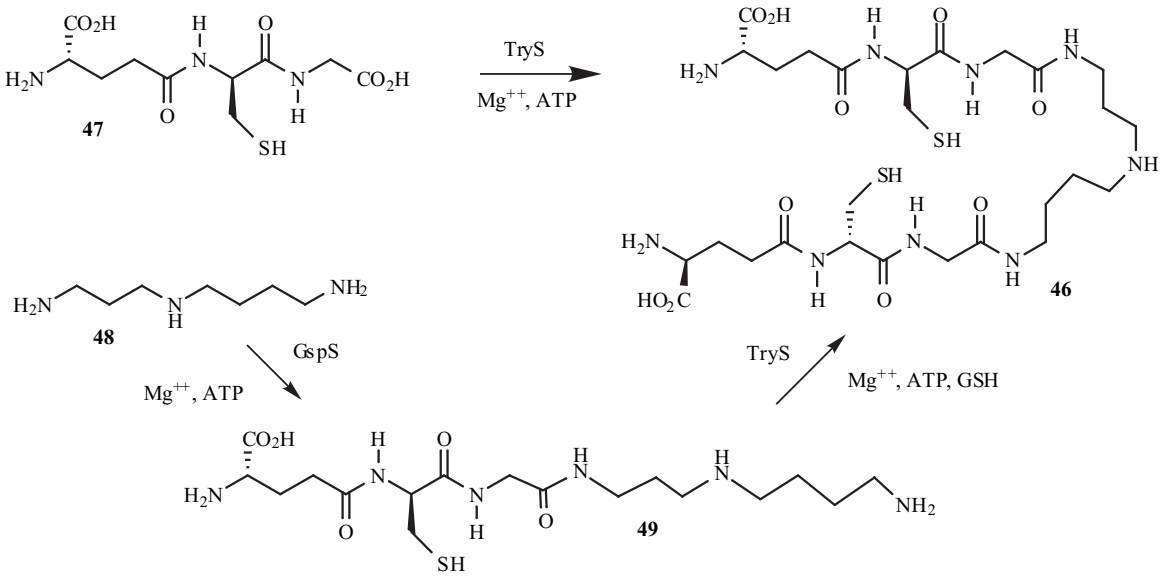
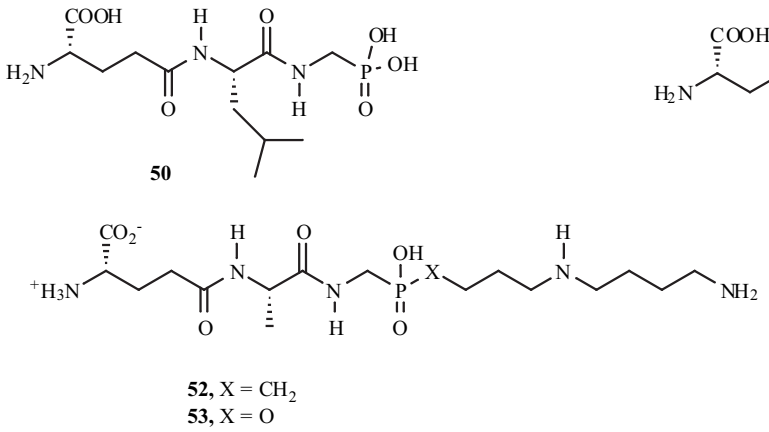


Fig. (14). Two possibilities for trypanothione biosynthesis.

was a 90 kDa protein, which had GspS activity and the other one was a 82 kDa protein that showed TryS activity. Flohé *et al.* also purified and characterized GspS using a different method. They reported a molecular weight of 78 kDa, conflicting with the data previous purification [173]. Molecular weight was not the only contradictory result, while Fairlamb *et al*. found amidase activity in GspS, Flohé *et al*. did not [174]. Tetaud and Fairlamb first tried to clone and express both enzymes in *E. coli* and *Saccharomyces cerevisiae*. Genes that encode GspS and TryS were identified in *C. fasciculata*. Molecular weights of purified proteins were in agreement with native ones, but no activity was observed for any of these proteins [175]. Based on previous studies on GspS in *E. coli*, Cys-79 was mutated to Ala-79 in the *C. fasciculata* enzyme. The resulting protein retained the synthetase activity without hydrolyzing glutathionylspermidine [176]. These results suggest that two different catalytic domains are present in this enzyme; nevertheless, how the parasite avoids futile synthetase/amidase cycling with net ATP hydrolysis caused by opposing to catalytic functions remains unsolved. However, prospects in searching molecular targets began to change substantially when *T. cruzi* TryS was cloned and expressed in *E. coli*. This protein was not only able to synthesize trypanothione from glutathionylspermidine and glutathione, but also from spermidine and glutathione. In addition, amidase activity was found in this enzyme too, but this TryS could hydrolyze both glutathionylspermidine and trypanothione at the same rate, showing a marked difference with *C. fasciculata* GspS, which could not significantly hydrolyze trypanothione. At this point, no gene encoding a GspS could be identified in *T. cruzi*, so it was postulated that in this parasite a single enzyme catalyzed formation of trypanothione [177]. The dual catalytic function of *T.cruzi* TryS, also found in *T. brucei brucei* [178], led to reinvestigate *C. fasciculata* TryS catalytic properties. The enzyme was cloned and expressed in *E. coli* as an active protein that catalyzed trypanothione formation displaying also an amidase activity [179]. In addition, *L. major* TryS was cloned and expressed in *E. coli* [180]. On the other hand, a pseudogene that encodes GspS (*GSPS*) was identified in this parasite. This last finding suggests that an evolutionary link occurs to resolve the observed divergence between *C. fasciculata* and *Trypanosoma* spp [180]. It seems reasonable to hypothesize that an ancestral *GSPS* with a narrow substrate specificity may have undergone duplication and divergence into two independent genes (*GSPS* and *TRYS*). This is the case of the substrate-specific enzymes (GspS and TryS) found in *C. fasciculata*. *Trypanosoma* genome had only retained *TRYS*; therefore, TryS evolved into a broad-specificity enzyme superseding the need of two enzymes in trypanothione biosynthesis [180]. This broadening of substrate specificity has been previously observed in the *T. cruzi* enzyme, which can conjugate a variety of polyamine analogues with GHS [181].

Gsps and Trys Inhibitors

Contrary to the widely studied trypanothione reductase, the corresponding crystal structures of GspS and TryS are still unknown. This fact have circumscribed the search of inhibitors of the enzymatic activity against these enzymes to the classical chemical structure / biological activity relationship (SAR). *C. fasciculata* GspS was the first enzyme available in large quantities and the first existing model to evaluate potential inhibitors. Because of the peptidic nature of one of the GspS substrates, namely GSH, and in order to establish the relevance of each residue of glutathione tripeptidic moiety, a series of tripeptides structurally related to of glutathione were designed and prepared. Compounds in which the glutamic acid residue had been replaced by different amino acids were not substrates for the enzyme; therefore, its presence seemed to be essential for molecular recognition. On the other hand, the alkylation of thiol side chain increased the catalytic efficiency of the substrate; in this case, the size of the alkyl group did not have to be bulkier than a propyl group. In addition, substitution of the L-Cys residue by L-Val or L-Ile did not change substantially the catalytic efficiency of the substrate. Most interesting, when L-Cys was replaced either by L-Leu or L-Phe, or when glycine was replaced by L-Ala,



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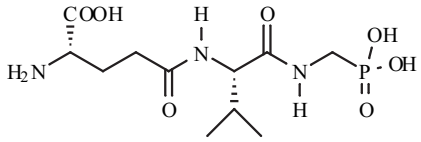


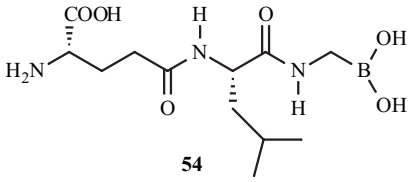
51

52, X = CH_2
53, X = O

Fig. (15). GSH and glutathionylspermidine analogues.

inhibition of the enzymatic activity was observed [182]. Based on these studies, phosphonic and phosphinic acid analogues of glutathione were designed and synthesized [183]. The phosphonic acid derivative of γ-L-Glu-L-Leu-Gly (**50**) showed to be a linear non-competitive inhibitor of *C. fasciculata* GspS with a K_i of 60 μM. Replacement of L-Leu by L-Val yielded the less potent inhibitor **51** with a K_i of 290 μM [183]. Based on the structure of this pharmacophore, it was designed and synthesized phosphonate and phosphonamidate analogues of glutathionylspermidine [184,185]. In these compounds, a phosphorus-containing group replaces the amide moiety mimicking the tetrahedral transition state of the amide bond formation [184]. Phosphonates and phosphinates had already been used to mimic tetrahedral transition state of other C:N ligases and have afforded inhibitors for Ala-D-Ala ligase, glutamine synthetase and glutathione synthetase [186,187]. Compound **52** exhibits a K_i value of 3.2 μM for binding to free enzyme (GspS from *Escherichia coli*), and 7.8 nM for binding to enzyme-substrate complex E·I*, while compound **53** was much less potent than **51** showing inhibition constants of 6 μM and 14 μM for binding free enzyme and E·I*, respectively. (Fig. **15**).

Taken γ-L-Glu-L-Leu-Gly as a lead structure, a new series of tripeptides analogues where the carboxylic moiety of glycine was replaced by different groups were synthesized. These compounds where tested against wild-type and C79A mutant *C. fasciculata* GspS. The boronic acid derivative



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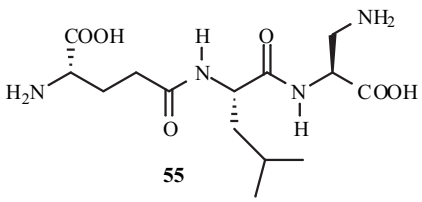


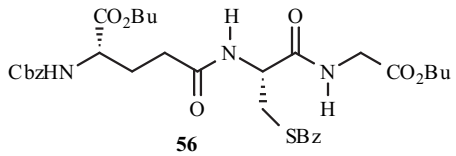
55

Fig. (16). γ-L-Glu-L-Leu-X derivatives.

(**54**) was an interesting drug, which presented a K_i of 81 μM [188]. Esters, amides and hydroxamic acids derivatives behaved as substrates and were also hydrolyzed by the amidase domain of the wild-type enzyme [188]. When the glycine residue was replaced by aminoacids contaning a nucleophilic side chain the efficacy was later improved. Specifically, γ-L-Glu-L-Leu-L-Dap (**55**) had a K_i of 7.2 μM against *C. fasciculata* GspS [189] (Fig. **16**).

Growth Inhibition Assays

Only few glutathione derivatives were tested against parasite cell cultures. D'Silva *et al*. investigated the *in-vitro* antiprotozoal activity of several N, S and COOH-blocked glutathione derivatives against *T. cruzi*, *T. brucei* and *L. donovani*. The most potent compound (**56**) presented an IC_{50} value of 1.9 μM against *T. brucei* [190,191]. This result suggested that protecting groups would facilitate the entry across the cell membrane and subsequent hydrolysis would occur by endogenous amidases and estearases to yield several *S*-blocked glutathione monoesters or free acids that would be recognized by the protein targets. Similarly, amide



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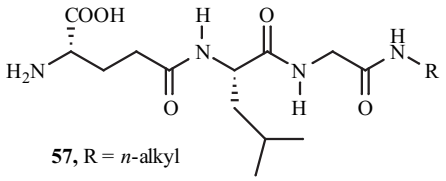
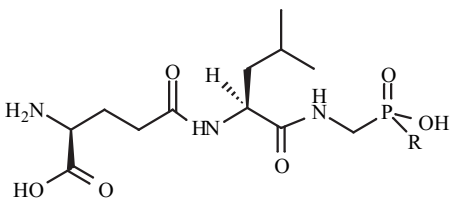


57, R = *n*-alkyl



58, R = *n*-alkyl

Fig. (17). Designed peptoids as antiparasitic agents.

derivatives of glutathione like L-γ-Glu-L-Leu-Gly tripeptide of general formula **57** were synthesized and tested against *T. cruzi* epimastigotes by Ravaschino *et al.* but no inhibitory activity was detected at a concentrations up to 20 μg/ml [192] (Fig. **17**). However, phosphinopeptides of general formula **58**, which were designed as simple pharmacophore on the basis of the chemical structure of drug **52**, resulted potent inhibitors against intracellular *T. cruzi* [193].

Trypanothione Reductase

Trypanothione reductase (TR) is a crucial and distinctive enzyme in trypanosomatids. TR activity was first discovered in crude extracts of *C. fasciculata* at the same time that trypanothione was isolated. Purification of this extract yielded a homodimeric flavoprotein, which used NADPH as cosubstrate. The flavin released by thermal denaturation was identified as FAD. Two residues of cysteine are present at the active site. Trypanothione reductase was purified from *T. cruzi* [194], and *L. donovani* [195]. The enzyme was cloned, overexpressed and purified form *T. cruzi* [199], *T. congolense* [197] and partially purified from *T. brucei* [198].

Contrary to GspS and TryS, TR has a counterpart in mammals: Glutathione reductase (GR). Both of these enzymes have the same metabolic function, converting a disulfide functionality into two thiol groups. TR and GR also share structural features such as a FAD prosthetic group, homodimeric subunits of approximately 50000 Da, and a Cys/Cys pair that is reduced and oxidized during catalysis. In order to determine if TR was a valid target for drug design, a comparison between TR and GR substrate specificities was carried out because of the likeness of both enzymes and their function. Despite the 40% aminoacid identity there was a high degree of substrate specificity.

Active Site and Substrate Specificity

Prior to crystal structure determination and site-directed mutagenesis studies, a structure-activity relationship evaluation was conducted on *C. fasciculata* TR. Various disufide synthetic derivatives structurally related to glutathione were tested as TR substrates. It was found that the spermidine bridge between both glutathione moieties was essential for molecular recognition; however, the macrocyclic structure of oxidized trypanothione was not. In addition, the presence of a hydrophobic section and also a positively charged amino function were required for a molecule to be substrate [199]. Some analogs of γ-Glu-L-Cys-dimethylaminopropylamide were synthesized and tested as substrates for TR. The α-amino group was more important than the α-carboxyl group for binding [200]. None of the above mentioned substrates were recognised for human GR. Then, it was considered that TR and GR might have common aminoacids involved in recognition of the glutamylcysteinyl moiety of their respective substrates. In fact, of 19 amino acid residues present in GR close to the binding site of GSSG, only 5 are not conserved in TR. Three arginine residues in human GR are replaced by neutral or acidic residues in TR. These alterations correlates well with different charges of disulfide substrates at physiological pH (–2 for GSSG and +4 for TS_2) [201]. Substrate specificity of TR and GR was further investigated by site-directed mutagenesis. Glu-18, and Trp-21 were crucial residues for TR substrate specificity. On the other hand, it was easier to adapt GR to TS_2 reduction than TR for GSSG reduction [202]. The crystal structure of TR from *C. fasciculata* [203] and *T. cruzi* [204], and binary complexes with Gsp_{ox} [205], NADPH, and $NADP^+$ [206], helped to analyze TR substrate specificity in spite of the analogies between GR and TR. Although the close structural resemblances between TR and GR, the former shows a wider active site. It was postulated that because GR-GSSG forms many polar interactions with the protein, the more open active site in TR, probably prevents tight binding of smaller GSSG molecule [207]. In conclusion, the most important causes of substrate specificity in both enzymes are attributed to the width and charge distribution at the substrate binding site.

TR Inhibitors

Availability of purified enzyme and information about its 3D-structure have consequently derived in a bulky number of TR inhibitors of different types.

Covalent Inhibitors

As mentioned before, arsenical drugs have been extensively used in the treatment of African trypanosomiasis. These drugs are potent inhibitors of TR *in vitro* by reaction with cysteine residues present at the active site of the enzyme. For example, melarsen oxide (**59**) forms an adduct with trypanothione that acts as a competitive inhibitor of TR [208]. Other arsenical drug like melarsoprol (**4**), inhibits one of the parasite adenosine transporters and reduces, in consequence, levels of FAD and NADPH leading to block $T(SH)_2$ biosynthesis [209]. Selectivity of these kind of compound is very low toward TR and they result highly toxic causing severe side effects. Similarly to arsenicals, (terpyridine)platinum(II) complexes (**60** and **61**) are irreversible inhibitors of TR activity targeting TR

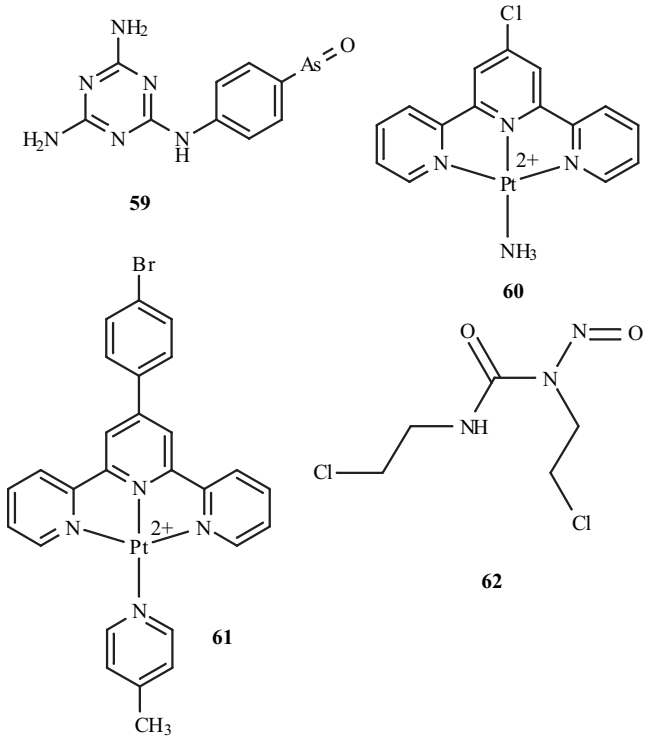


Fig. (18). Covalent inhibitors of TR activity.

cysteinyl residues. In this case, selectivity was not so good due to **60** and **61** behave as reversible inhibitors of this enzyme. Nevertheless it is known that they are able to intercalate and/or platinate DNA and react with cysteinyl and histidyl residues of other proteins [210]. Finally, nitrosoureas as carmustin (**62**), also form covalent bonds to TR by carbamoylation of the cysteine thiol at the active site. (Fig. **18**).

Competitive Inhibitors

As depicted before, antimonials have been widely used for the treatment of leishmaniasis. Antimonial (Sb^{5+}) preparations are prodrugs of Sb^{3+}. *In vivo* conversion of Sb^{5+} into Sb^{3+} still remains unclear, but it has been demonstrated that Sb^{3+}, reversibly inhibits TR *in vitro*. Trivalent sodium antimonyl gluconate (triostam) resulted in a linear competitive inhibitor towards TR activity; unfortunately, this drug also behaved as an inhibitor of human GR activity [211]. Polyamines analogues and tricyclic compounds were interesting examples of competitive inhibitors. Tricyclic compounds, which were known antidepressant and antipsychotic agents, proved to be potent inhibitors of TR activity. Clomipramine (**63**) was the most potent drug among 30 phenothiazine and tricyclic antidepressants, with a K_i of 6 μM [212]. They showed a linear competitive inhibition employing trypanothione as the substrate, while exhibited a non-competitive inhibition if employed NADPH. Although these compounds presented undesired side effects for the mammalian host, they proved to be an interesting model for binding studies. The knowledge of TR crystal structure and the biological activity of these compounds aided at revealing some characteristics of the enzyme binding site. The tricyclic moiety would fit the hydrophobic pocket of TR binding Leu-17, Trp-21, Tyr-110, Met-113 and Phe-114. The *N,N*-dimethylaminopropyl group was close to Glu-466 and Glu-267 residues. In order to study the influence of a positive charge at the side chain on biological activity the *N,N*-dimethylaminoalkyl moiety was replaced by a carboxylic function. This structural variation resulted in a dramatically loss of potency against *C. fasciculata* TR, but not against human GR. Chlorpromazine (**64**) and analogues **65** and **66** showed a K_i of 14, 68 and 24 μM, respectively, against TR while the carboxylic analogue **67** showed a K_i of 1,400 μM [213]. Acridines, which display a wide range of antitumor, antibacterial and antiparasitic activity, were another interesting inhibitors of TR activity. Mepacrine (**68**) arose as the main member for this type of drugs. All of these compounds showed specific inhibition against parasitic TR; however, the antiparasitic activity did not correlate with the TR inhibitory activity exhibited by these drugs [17] (Fig. **19**).

The polyamine and antihypertensive agent kukoamine A [N^1,N^{12}-bis(dihydrocaffeoyl)spermine] inhibited TR activity as a mixed inhibitor (K_i = 1.8 mM, K_{ii} = 13 mM), and it showed no significant inhibition of human glutathione reductase (K_i > 10 mM). Synthetic N^1,N^8-bis(dihydrocaffeoyl)spermidine proved to be a competitive inhibitor with K_i = 7.5 mM. In order to analyze the influence of chain length on inhibitory potency, a series of mono- and di-acylated spermines and spermidines were synthesized [214]. Other series of selectively blocked spermines and spermidines were synthezised and tested against TR. Polyamines blocked with hydrophobic groups were potent inhibitors of TR activity. For example, phenylpropyl and naphthyl derivatives **69**, **70** and **71** exhibited K_i values of 3.5 μM, 5.5 μM and 9.5 μM, respectively. This enzymatic activity correlated quite well with trypanocidal action exhibited by these drugs against four *T. brucei* spp. strains with IC_{50} values ranging from 0.19 to 0.83 μM [215]. Bearing in mind these results, some structural variation were conducted on compounds **69–71** employing 2-amino-diphenylsulphides and phenothiazines as hydrophobic moieties instead of the phenylpropyl and naphthyl groups. Of the 30 synthesized spermine and spermidine derivatives, **72** was the most relevant inhibitor with a K_i of 0.4 μM against TR activity [216]. Polyamine derivative **73**, which was selected by screening of a spermidine-peptide conjugate library, showed a K_i of 100 nM [217]. The mechanism of action of these compounds was investigated and, surprisingly, **72** and **73** were non-competitive inhibitors of TR [218] (Fig. **20**).

Crystal violet (**3**) was a potent inhibitor of *T. cruzi* trypanothione reductase *in vitro*. In this case, the inhibition was competitive with respect to trypanothione (K_i = 5.3 μM). However, crystal violet was not able to decrease the level of reduced thiols in intact cells [219].

Turn-Coat Inhibitors

Turn-coat inhibitors, also known as subversive substrates, are compounds that bind the catalytic site of TR and are reduced by NADPH in a one electron transfer reaction. They are subsequently reoxydized by O_2 to produce

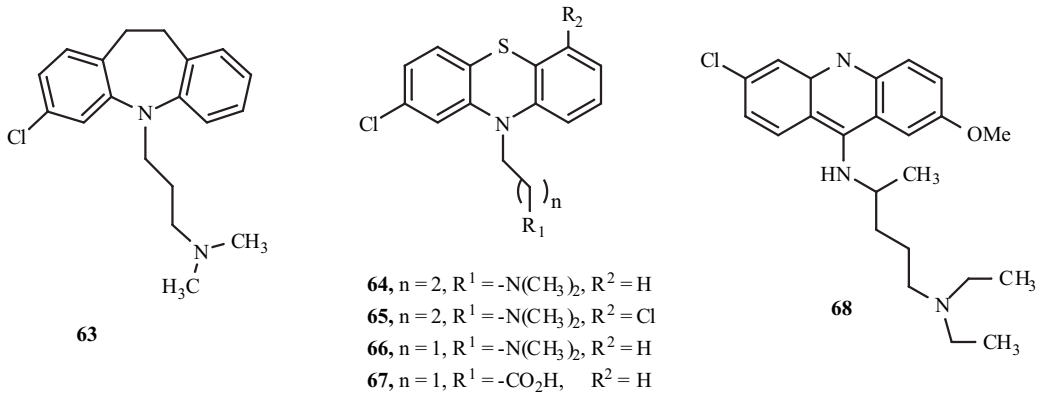
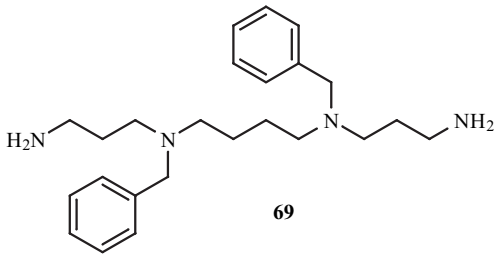


Fig. (19). Tricyclic derivatives as inhibitors of TR activity.



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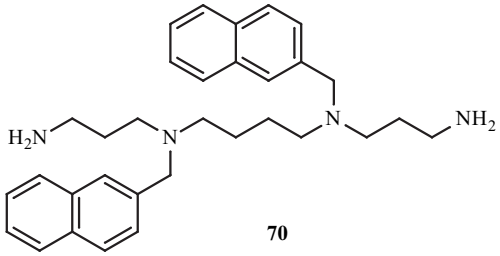

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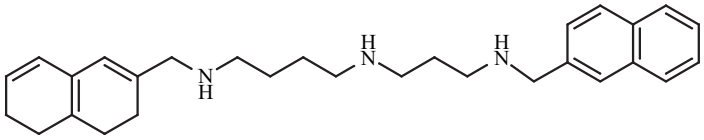

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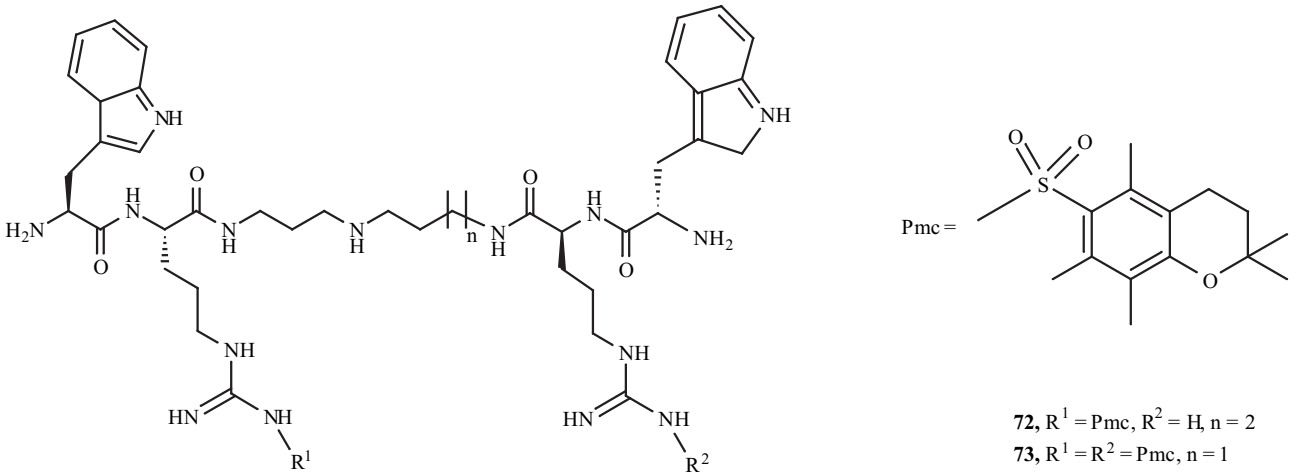
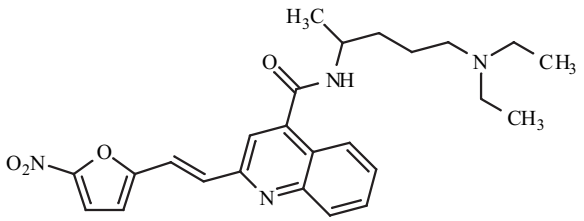


Fig. (20). Polyamine derivatives as inhibitors of TR activity.

superoxide anion radicals that inhibit the reduction reaction of the natural substrate in a redox cycle. This process generates reactive oxygen species and a net consumption of NADPH and oxygen. The nitrofuran derivative nifurtimox (**1**) is a turn-coat inhibitor, while chinifur (**74**) was a very potent nitrofurane towards TR [220]. In addition, the nitroimidazole derivative benznidazole (**2**), other well-known antiparasitic drug, is also a redox cycler compound. The mode of action of nifurtimox and nitroimidazoles such as metronidazole and megazol (**75**) was investigated. Contrary to nifurtimox and metronidazole, TR was not the target for megazol [221]. Further investigations indicated that nitrofurans behaved as redox cyclers inhibitors, whereas megazol was a thiol scavenger, particularly, for trypanothione [222] (Fig. **21**).

Taking into account that some quinones could act as redox cycler inhibitors, a series of naphthoquinones bearing acidic and basic side chains were designed, synthesized and evaluated as TR redox cyclers inhibitors. Compounds having basic side chains such as **76** and **77** were able to



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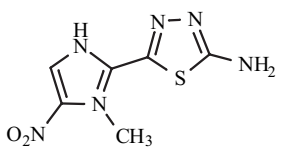

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Fig. (21). Nitrofuran and nitroimidazole inhibitors.

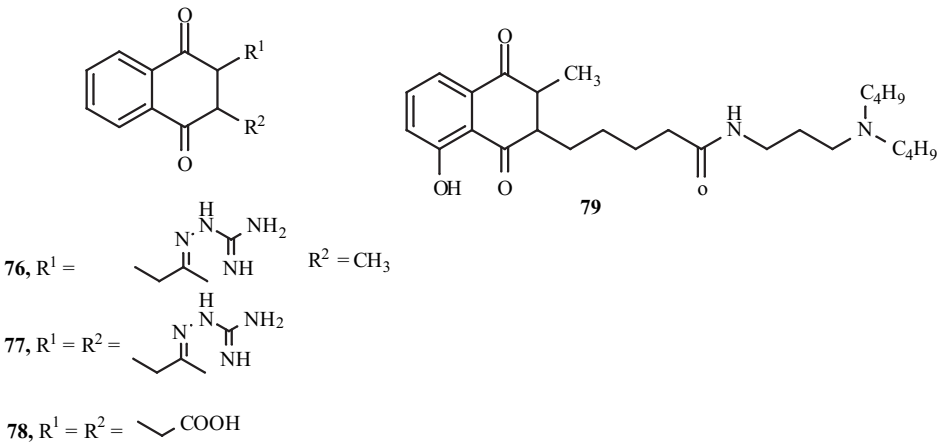


Fig. (22). Naphthoquinone inhibitors.

oxidize several molar equivalents of NADPH, while those possessing acidic side chains such as **78** exhibited vanishing action [223]. A library of 1,4-naphthoquinones derivatives was produced by parallel synthesis and the resulting compounds were evaluated against *T. cruzi* TR activity. Of the 1360 amides constructed from combination of 12 newly synthesized 1,4- naphthoquinone carboxylic acids with 120 amines, **79** emerged as the most potent drug with an IC_{50} value of 0.3 μM [224] (Fig **22**). Many other naturally occurring and synthethic naphthoquinones and anthraquinones were reported to possess antitrypanosomal activity. Crystal violet is another example of a chemoprophylactic agent that also acts as a redox cycler drug [225]. Despite the effectiveness of these kind of TR inhibitors, selectivity towards other redox active enzymes is very poor; therefore, prospects for these TR inhibitors are not very promising.

Dihydrofolate Reductase

Dihydrofolate reductase (DHFR) catalyses the reduction of dihydrofolate to tetrahydrofolate. Tetrahydrofolate is methylated to form methylene tetrahydrofolate, which is a vital cofactor to convert deoxyuridine monophosphate into thymidine monophosphate. DHFR constitutes an interesting target for drug design, because of DHFR inhibition prevents biosynthesis of thymidine, leading to cell death [226]. In addition, the leishmanial and trypanosomal DHFR and the corresponding human enzyme show structural differences [227]. A selective inhibition of leishmanial and trypanosomal DHFR would lead to growth impairing of the parasite because these microorganisms do not have a mechanism of transport of this cofactor from the host.

Most known DHFR inhibitors contain a heterocyclic pyrimidine or triazine ring with amino substituents at the 2- and 4-positions. In 1988 it was reported that some 5-benzyl-2,4-diaminopyrimidines were selective inhibitors of leishmanial DHFR activity [228]. These data correlated quiet well with the cellular activity exhibited by these compounds against *L. donovani* amastigotes. Some years ago, a chemical structure / biological activity SAR study was carried out on 5-benzyl-2,4-diaminopyrimidines against leishmanial and trypanosomal DHFR [229,230]. The inhibition of DHFR activity by compounds of general formula **80**–**82** was explored (Fig. **23**). Some of these compounds were potent inhibitors of DHFR activity. An alkyl chain length of 2-6 carbon atoms at the 3'-position showed maximum enzyme activity and selectivity. For example, **80a** (K_i = 23 nM for *T. cruzi* DHFR), **80b** (K_i = 3.6 nM for *T. brucei* DHFR), **80c** (K_i = 65 nM for *L. major* DHFR) [229]. The maximum activity against parasite cell not always correlate with the more potent enzymatic inhibitors. Long chain derivatives (8-12 carbon atoms) were the most potent cell growth inhibitors [229]. 4'-Mono and 3',4'-disubstituted derivatives **81** and **82** were potent drugs but to a lesser extent than **80** [230]. However, none of the tested compounds showed any *in vivo* activity against Chagas' disease.

Novel parasite DHFR inhibitors, in which the pyrimidine ring was absent, were designed, synthesized, and biologically evaluated towards *T. cruzi* and *T. brucei rhodesiense* DHFR activity [231]. Compounds **83**, **84**, and

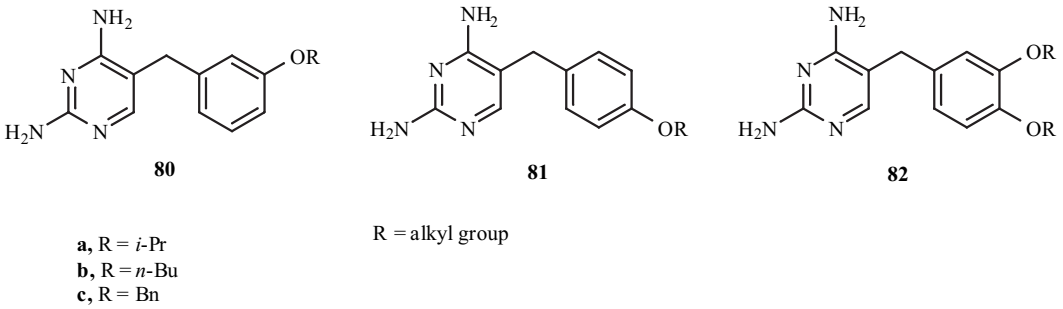


Fig. (23). 5-Benzyl-2,4-diaminopyrimidines as inhibitors of leishmanial and trypanosomal DHFR activity.

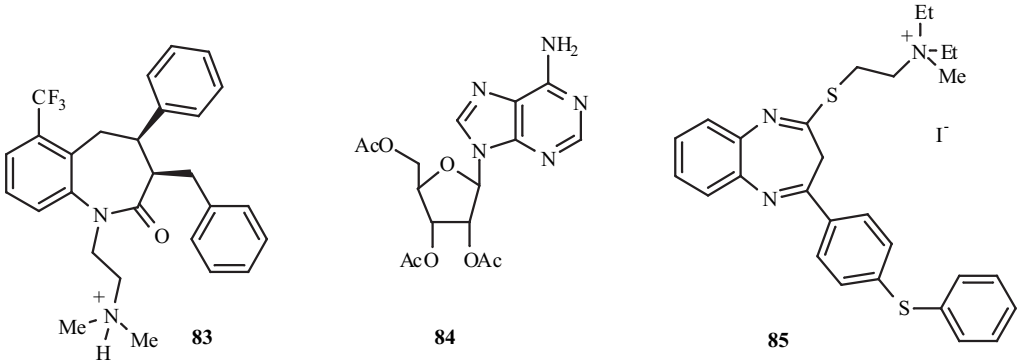


Fig. (24). Selected inhibitors of *Tc* and *Tb*DHFR activity.

85 exhibited a moderate efficacy as inhibitors of DHFR [231] (Fig. **24**).

Compound **83** showed some selectivity for the parasite enzyme. Compounds **83** and **84** exhibited a modest potency against *T. cruzi* (MIC 54 μM and 55 μM, respectively) and high efficacy against *T. brucei rhodesiense* at the low micromolar range (IC_{50} = 3.6 μM and 1.0 μM, respectively) [231].

The dihydrofolate reductase is also a validated target for antimalarial agents. Pyrimethamine (**86**) and cycloguanil (**87**) are common antimalarial antifolates, but their utility is limited because of resistance of *P. falciparum* strains [232–234] (Fig **25**). In order to explain the structural bases of antifolate resistance, the concept of the "steric constraint hypothesis" was presented [235]. Therefore, several DHFR inhibitors lacking the substituents at positions where steric clash was most likely were evaluated [236]. These drugs resulted in a significant impairing of resistance [235,237]. It is known that more flexible compounds than **86** and **87** inhibit the enzymatic activity of resistant mutant *Pf*DHFRs [237].

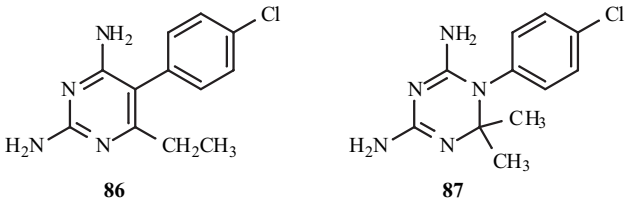


Fig. (25). Chemical structure of antifolates pyrimethamine and cycloguanil.

Based on a combined pharmacophore screening-molecular docking strategy, twelve new compounds not structurally related to conventional antifolates have been identified [238]. These compounds inhibit the enzymatic activity of the wild type and resistant *Pf*DHFRs. In addition, a 3D-pharmacophore model with the ability to predict enzymatic inhibition of *Pf*DHFR have also been developed [239]. A pharmacophore model with five features is generated: two hydrogen bond donors, one positive ionizable center, one hydrophobic aliphatic center and one hydrophobic aromatic center. The use of this approach allowed to estimate quantitatively the activity of a large number of compounds in a short period of time [239]. 2,4-Diamino-5-methyl-6-(aryl)thiopyrrolo[2,3-*d*]pyrimidines derivatives were designed and synthesized as DHFR inhibitors of opportunistic pathogenic parasites that afflict patients with AIDS. Some of these compounds were potent and selective against *T. gondii* DHFR activity compared to mammalian DHFR. For example, compound **88** was 16-fold more efficient and equally selective against *T. gondii* DHFR as the clinically employed trimethoprim [240] (Fig. **26**).

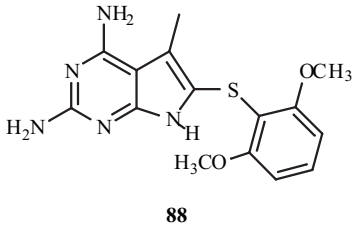


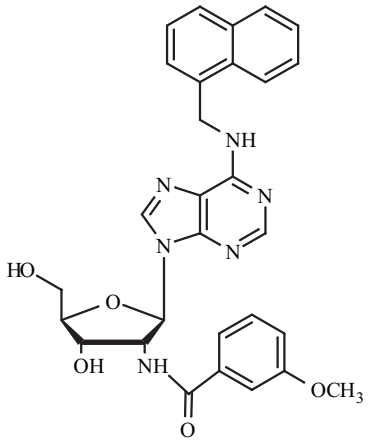
Fig (26). Selected inhibitor of *Tg*DHFR activity.

Glyceraldehyde-3-Phosphate Dehydrogenase

This is another interesting enzyme to be employed as a valid target for drug design. Rigorous studies on energy metabolism in *T. brucei* have established that the bloodstream form depends solely on glycolysis with the excretion of pyruvate for energy production [241,242]. There is evidence that, unlike in mammalian cells, glycosomal glyceraldehydes-3-phosphate dehydrogenase (GADPH) exerts significant control over the glycolytic pathway. The reaction mechanism consists in a multistep catalytic process [243]. GADPH catalyzes the oxidative phosphorylation of glyceraldehyde 3-phosphate (GAP) into 1,3-biphospho-D-glyceric acid (1,3-diPG). Inorganic phosphate (P_i) and nicotinamide adenosine dinucleotide (NAD^+) are involved in the activity and the [NAD^+] / [NADH] ratio needs to be mantained constant within the cell. In *Leishmania* spp. and in *T. cruzi* glycolysis is always active and biochemical studies on *T. cruzi* cell suggested that carbohydrate catabolism is its major energy source. Thus, selective inhibition of parasite glycolytic enzymes over the mammalian enzymes, would provide an interesting approach for chemotherapy of tropical diseases produced by *T. cruzi* and *Leishmania* spp.

X-ray studies of structures of *T. brucei*, *T. cruzi*, and *L. mexicana* [244,245] show the binding mode of the adenosyl moiety of the NAD^+ cosubstrate and have led to the design of adenosine analogues as inhibitors of trypanosomatid GADPH's [246,247].

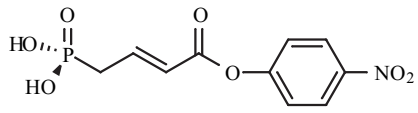
The adenosine scaffold provides several chances for improving affinity and selectivity. Specifically, a hydrophobic gap protruding from the ribosyl C-2' was a trypanosomatid GADPH's feature. This "selectivity gap" is not present in human DHFR due to a difference in protein backbone conformation. N^6-(1-naphthalenemethyl)-2'-deoxy-2'-(3-methoxybenzamido)adenosine (**89**) is a trypanosomatid-selective GADPH inhibitor with IC_{50} values of 25 μM, 12 μM, and 6 μM for *T. brucei*, *T. cruzi*, and *L. mexicana* GADPH's, respectively [247] (Fig. **27**).



89

Fig. (27). Chemical structure of a representative adenosine derivative as inhibitor of GADPH activity.

A structure/activity study for a series of benzamides with structural variations at the naphthyl group was carried out considering new synthetic approaches for the synthesis of N^6-(substituted)-2'-deoxy-2'-(amido)adenosine, N^6-(substituted)-5'-deoxy-5'-(amido)adenosine and 2',5'-dideoxy-2',5'-(bisamido)-adenosine analogues [248]. There was a strong evidence to believe that exist a covalent bond between the enzyme and the substrate through the cysteine residue of the GADPH [249]. On the basis of this idea, and in order to introduce new mechanistic insights, compounds bearing an electrophilic group such as an epoxide group or an α-enal group were designed and synthesized, which proved to be potent GADPH irreversible inhibitors [249]. Compounds bearing a *p*-nitrophenol ester group were synthesized. These compounds reacted with a cysteine residue at the active site to form a thioacyl enzyme releasing a *p*-nitrophenate unit, which could be easily monitored [249]. Compounds **90** and **91**, which are GAP analogues, give a time-dependent inactivation of glycosomal *T. cruzi* GADPH (Fig **28**). The irreversible effect was confirmed by the fact that no enzymatic activity was recovered after dilution of the enzyme/inhibitor mixture obtained after the incubation time. Since these irreversible inhibitors were Michael acceptors, two different ways of inactivation were possible: acylation of the cystein residue or 1,4 addition by the cysteine thiol function.



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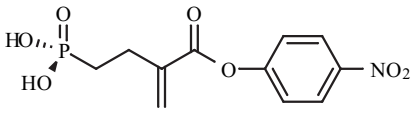
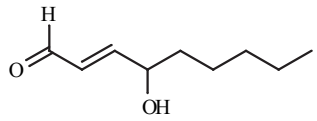


91

Fig. (28). Chemical structure of GAP analogues.

On the other hand, it is known that GADPH is highly sensitive to inactivation by lipid peroxidation products *in vitro* and *in vivo* [250]. Reactive aldehydes, such as keto aldehydes, 2-alkenals, and 4-hydroxy-2-alkenals, are able to covalently modify biomolecules and are considered important mediators of cell damage. (*E*)-4-Hydroxy-2-nonenal (HNE; **92**) is a major product of lipid peroxidation [251] and easily react with proteins sulfhydryl groups [252] (Fig. **29**). The modification sites in the enzymes produced by HNE has been identified by mass spectrometry, by using ESI-LC-MS and MALDI-TOF MS [253]. The covalent binding to HNE with GADPH was investigated [250]. These studies indicated that the loss of activity was attributable to covalent binding of HNE to three amino acid residues, cysteine, lysine, and histidine. Different experiments including MALDI-TOF MS analysis afforded evidences that the reaction between HNE and GADPH took place *via* a Michael reaction. LC-MS analysis showed that five amino acids contained the HNE Michael adducts at His-164, Cys-244, Cys-281, His-327, and Lys-331. However, Cys-149 at the active site did not undergo reaction with HNE suggesting that HNE inactivation of GADPH is due to changes of the surface amino acids. The catalytic center was untouched [254].



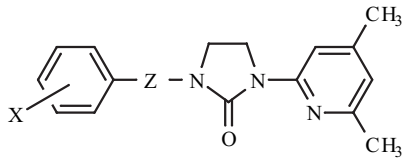
92

Fig. (29). Chemical structure of (*E*)-4-Hydroxy-2-nonenal.

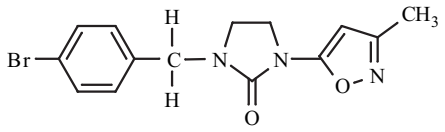
Leishmaniasis

As previously mentioned, leishmaniasis is a widespread parasitic disease, which is caused by protozoan parasites of the genus *Leishmania* affecting million of persons worldwide [38,39]. As chemotherapy for leishmaniasis is still deficient, a huge amount of work has been carried out to solve this problem.

In the search for new antileishmanial agents, many efforts have been conducted. For example, arylcarboxamides derivatives of 2-amino-4,6-dimethylpyridine showed IC_{50} values at the low micromolar range against *in vitro* extracellular promastigotes of *L. donovani* and *L. braziliensis* [255,256]. On the basis of these results, new imidazolidin-2-one derivatives of 2-amino-4,6-dimethylpyridine were designed and prepared (Fig. **30**). Compounds **93** and **94** were potent inhibitors against the intracellular form of the parasite (IC_{50} 13 μM and 7.0 μM, respectively). Contrary to common antileishmanial drugs,



93, X = 2-Br, Z = CH_2
94, X = 4-CH_3, Z = SO_2



95

Fig. (30). New imidazolidinone derivatives as antileishmanial agents.

the intracellular form was more sensitive to **93** and **94** than the promastigote form of the parasite. These results led to the preparation of new imidazolidinone derivatives. Compound **95** was an interesting drug, which exhibited an IC_{50} value of 9.5 mM against *L. infantum* promastigotes. This compound constitutes a new lead for the design of novel antileishmanial agents.

Recently, 1,4-diarylpiperazines were synthesized and evaluated against the *Leishmania* parasite [257]. It had been depicted that closely related anologues were shown to be antipneumocystic [258] and trypanocidal agents [259]. Substituted 1,4-diarylpiperazines by 4-benzamidine groups were designed and prepared. Derivatives bearing cyclic moieties such as cyclohexyl (**96**), or benzyl (**97**) showed potent inhibition (IC_{50} = 11.6 μM and 4.3 μM, respectively) against *L. donovani* respect to their parent diamidine (**98**) (IC_{50} = 21.5 μM), which was similar to pentamidine. 1,4-bis[4-(1*H*-benzimidazol-2-yl)phenyl]piperazine (**99**) showed a potency at the nanomolar level (IC_{50} = 410 nM), almost seven-fold more potent than pentamidine and as potent as amphotericin B in *in vitro* studies, constituting a promising candidate for the develop of new drugs. In addition, a possible relationship between the *in vitro* activity observed and DNA binding affinity of these diamidines was investigated, but a correlation was not observed yet [259] (Fig. **31**).

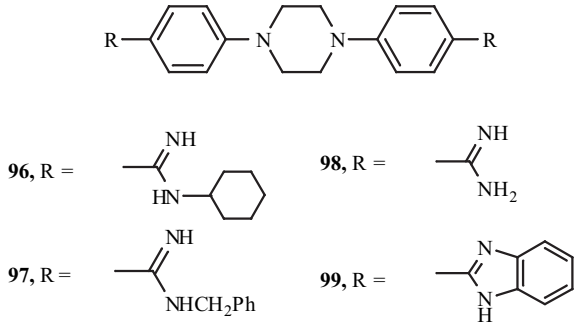
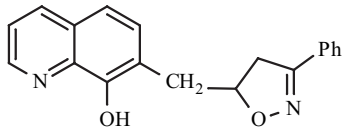


Fig. (31). 1,4-Diarylpiperazines as antileishmanial agents.

Recently, the first report about the antileishmanial activity of a new 8-hydroxyquinoline derivative has been described [260]. Diverse 8-hydroxyquinolines have been shown to possess antitumor activity [261]. 7-[5'-(3'-Phenylisoxazolino)methyl]-8-hydroxyquinoline (**100**) required similar concentrations than amphotericin B for IC_{50} against *L. tropica*, *L. major* and *L. infantum*. Although the IC_{50} values of amphotericin B are slightly better than those for **100**, this compound was signifincantly less toxic than amphotericin B. However, exact data on toxicity for **100** was not well established. Further studies on leishmanial effect and toxicity for optimization of 8-hydroxyquinoline derivatives are being conducted.

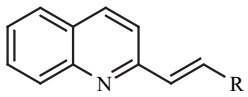


100

Fig. (32). Chemical structure of 7-[5'-(3'-phenylisoxazolino)methyl]-8-hydroxyquinoline.

2-Alkyl and 2-arylquinolines proved to have interesting pharmacological properties such as against *Leishmania* spp. [262], *Plasmodium* [263], *Trypanosoma* spp. [264] and also were found to inhibit the human immunodeficiency virus of type-1 targeting (HIV-1) integrase [265,266].

Recently, it was reported a study about antiprotozoal activity of several 2-substituted quinolines [267]. Among the quinolines analized, some new compounds were found to be promising lead drugs. Taken compound **101** as an example, this drug was very effective against *T. cruzi* (IC_{50} = 0.15 μM), and even more potent than nifurtimox (IC_{50} 0.45 μM). In addition, compounds **101** and **102** were more potent (IC_{50} 2.0 μM) than the reference drug glucantime (IC_{50} 7.15 μM) against the amastigote forms of *L. infantum*. Both of these drugs were as effective as miltefosine (IC_{50} 3 μM), the oral reference drug against the amastigote forms of *L. amazonensis*. However, cytotoxicity was observed against normal cells.



101, R = Br
102, R = CHO

Fig. (33). Chemical structure of quinoline derivatives.

Polyamines are essential for cell proliferation and differentiation; therefore, the blockade of parasitic biosynthesis of polyamines constitutes a valid chemotherapeutic approach. Taking into account that diverse polyamines derivatives present a broad range of pharmacological actions against several etiological agents of parasitic diseases, a synthesis of diamines derivatives on solid support was carried out [268]. These drugs were evaluated against *P. falciparum* D6 and chloroquine resistant W2 strain and *Leishmania* promastigotes of *L. donovani.* Among all polyamines studied, **103**-**109** were the most potent drugs against *P. falciparum* D6 and W2 strains and

103, n = 1, $R^1 = R^2$ = 3-MeO-4-BnO-benzyl
104, n = 2, $R^1 = R^2$ = 3-MeO-4-BnO-benzyl
105, n = 1, R^1 = 4-benzyloxy-benzyl, R^2 = Bn
106, n = 2, R^1 = 4-benzyloxy-benzyl, R^2 = Bn
107, n = 1, R^1 = H, R^2 = 3-phenyl-propyl
108, n = 1, R^1 = H, R^2 = 4-bromo-benzyl
109, n = 2, R^1 = H, R^2 = 2,3,4,5,6-pentafluoro-benzyl

Fig. (34). Chemical structure of polyamines derivatives.

L. donovani. The fact that these compounds were effective against two different parasites lead to consider that this family of drugs might be potentially useful as broad-spectrum antiparasitic agents (Fig. **34**).

Toxoplasmosis

Toxoplasmosis is a major parasitic disease caused by the apicomplexan parasite *T. gondii* [269]. This disease is transmitted by eating cysts present in undercooked meat or by intake of sporulated oocysts from infected soil [270]. One of the treatments against *T. gondii* infections take advantage of the synergistic efect of pyrimethamine, which inhibit the enzymatic activity of dihydrofolate reductase, and sulphonamides, which block dihydrofolic acid synthetase [271]. However, this combined pyrimethamine-sulphadiazine therapy is frequently associated to toxic side effects such as: haematological toxicity caused by pyrimethamine and cutaneous rash, leucopenia and thrombocytopenia provoked by sulphadiazine.

The inhibitory effect of azasterols against *T. gondii* profileration have been explored [272]. As mentioned before, azasterols are known inhibitors of $\Delta^{24(25)}$-sterol methyltransferase, a key enzyme in ergosterol biosynthesis. These type of drugs exhibited selective antiproliferative effects against trypanosomatid parasites [273–275]. 22,26-Azasterol and 24,25-(*R*,*S*)-epiminolanosterol have also shown potent and selective antiproliferative activity against *T. gondii* tachyzoites. The precise molecular mechanism of these compounds remains unclear. 24-Alkyl sterols have not yet been identified in *T. gondii*; however, it would be possible that the selective accumulation of these compounds in parasite membranes such as the mitochondria could alter their function. In addition, a synergistic effect is observed when a combination of pyrimethamine-sulphadiazine therapy with azasterols is used [276]. A significant improvement of their antiproliferative activity is observed. The observed synergism suggests a dramatic lowering of antifolates doses keeping the same antiproliferative effect.

CONCLUSIONS

The aim of this review was focused at presenting a broad scope of several attractive targets for rational design of drugs to combat several tropical protozoan parasitic diseases. Trypanosomiases and leishmaniasis can be considered as neglected diseases presenting insignificant profits for investment from pharmaceutical companies. All of these diseases constitute at the present time an important cause of morbidity and mortality mostly in developing countries due to their occurrence is strongly associated with poverty. Although there are other potentially valuable targets, only the more representative ones were treated in this article. However, only with the appropriate knowledge of the biochemistry and physiology of the involved parasite will be possible the eradication of all of these parasitic diseases.

ACKNOWLEDGMENTS

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